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Study of some Immunological Marker and Antimicrobial Susceptibility on Clinical Isolated from Patients with Otitis Media

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By

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

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Dedication

To …

My Mother and my Father.

My Brothers, My Sisters, My Children and

My Friends.

To everyone who helps and supports me.

I Dedicate this work.

Anas 2022

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Summary

Otitis media is a health problem for children as well as adults and the main cause of hearing loss, full deafness, facial paralysis, brain abscess, internal ear infection, and lining membrane inflammation.

The study includes a collection and laboratory culture of samples (middle ear swabs). From ninety patients, forty-eight male and forty two female with middle ear infection, affended at Al- Hila Teaching Hospital (ENT unit) and Imam Sadiq Hospital (ENT department) in Babylon city during the period between (October 2021 to February 2022). To investigate the microbial pathogen, blood samples were collected from all patients and thirty apparently healthy as control to separate, serum samples for cytokine (IFN-γ, IL-10) and Human Cytotoxic T Lymphocyte Associated Antigen 4 (CTLA-4) estimation, the isolates were identified according to cultural, biochemical tests, confirmed by VITEK 2 system compact and molecular diagnosis method, the results for positive culture were only (64) patients versus (26) patients revealed negative culture.

The patients were distributed into eight groups according to the age, the results showed that the highest rate of infection was in the first age group (1 – 9 years) at the rate of (16.7%).

It was found in this study that the most causative agents are bacterial infection at rate of64 (71.1%) out of 90 samples, the most common types of bacterial isolated were it is Staphylococcus aureus (34) isolates (37.8%), Staphylococcus epidermidis (6) isolates (6.7%) and Streptococcus pyogenes (1) isolates (1. 2%), while Pseudomonas aeruginosa (7) isolates (7.8%), Escherichia coli and, Klebsiella pneumoniae (4) isolates (4.5%), Enterobacter cloacae (3) isolates (3.4%), Proteus mirabilis and Acinetobacter ursingii (1) isolates (1.2%).

Fungal agents 15 (16.7%) out of 90 samples were have three genera *Aspergillus niger* (1) (1.2%), *Candida parapsilosis* (10) (11.2%)and Malassezia furfur (4) (4.5%).

The antibiotic disc diffusion test was done using (9) different clinically important antibiotics.

The result showed that Ceftriaxone and Erythromycin antibiotics was highly effective against Gram positive bacteria (73.5%) and (70.5%) respectively, while Amikacin and Gentamicin antibiotics were more effective against Gram negative bacteria (85.7%) and (71.4%) respectively.

The results of the immunological examination of some immunological parameters for the patients serum showed significant differences compared with the control group, the mean of IL-10 concentration to patients serum level mean ± SD 28.59±0.9 pg/ml were a significant decreased compared with the control group mean ± SD 43.07±1.9 pg/ml special in fungal causes mean ± SD 27.82±1.8) mg/l, in unknown causes mean ± SD 27.12±1.5 mg/ml and bacterial causes mean ± SD 29.90±1.6 mg/l, comparison with control group mean ± SD 43.07±1.9b mg/l .

the mean of IFN-γ concentration to patient's serum level (68.35±2.6) pg/ml were a non-significant increase compared with the control group (61.93±2.5) pg/ml special in fungal causes group 75.03±5.5 pg/ml, unknown causes group 70.04±5.2 pg/ml and significant increase in bacterial causes group 64.68±3.4 pg/ml in compared with control 61.93±2.5 a pg/ml.

The study showed that the immune system of the patients was affected by the bacterial infection, which recorded the highest rate of the disease.

the mean of Human Cytotoxic T Lymphocyte Associated Antigen 4 (CTLA-4) concentration in patients 42.99±1.4ng/ml compared with control 34.03±1.7 ng/ml. was a significant increased $P < 0.05$ special in fungal causes groups 43.79±2.2 ng/ml and bacterial causes group 42.48±1.8 ng/ml comparison with control group 34.03±1.7 a ng/ml.

The study showed that the immune system of the patients was affected by the fungal infection bacterial infection in IFN-γ IL-10 and CTLA-4 , while in IL-10 showed that the immune system of the patients was affected by the bacterial infection in which recorded the highest rate of the disease

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List of Abbreviations

Symbol	Description
AOM	Acute otitis media
APCs	Antigen presenting cells
AIDS	Acquired Immunodeficiency Syndrome
BHIB	Brain Heart Infusion Broth
CNS	Central Nervous System
COM	Chronic otitis media
CSOM	Chronic suppurative otitis media
CD4	Cluster of differentiation 4
ºC	Degree Celsius
EAC	External auditory canal
ENT	Ear ,Nose and throat
ETS	Environmental tobacco smoke
ELISA	Enzyme Linked Immunosorbent Assay
EMB	Eosin Methylene Blue Agar
ET	Eustachian tube
GAS	Group A streptococcus
GM-CSF	Granulocyte-macrophage colony-stimulating factor
G-CSF	Granulocyte colony-stimulating factor
HIV	Human immunodeficiency virus
HMI	Humoral mediated immunity
H_2O_2	Hydrogen Peroxide
IL	Interleukin
IE	Inner ear
IFN-γ	Interferon gamma
IL-10	Interleukin - 10
IU	International Unit
LSD	Less Significant Difference
L	Liter
MRSA	Methicillin resistant *Staphylococcus aureus*
MCM	Mucous cell metaplasia
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
MR-VP Reagent	Methyl red-vogues proskauer reagent
ME	Middle ear
MEE	Middle ear effusion
MRI	Magnetic resonance imaging
Ml	Mililiter
Mg	Miligram
µL	Microliter
µg	Microgram

μL	Microliter
NCBI	National Center for Biotechnology Information
ng	Nanogram
O.D.	Optical Density
OM	Otitis media
OME	Otitis media with effusion
OE	Outer ear
P	Probability
PCR	Polymerase chain reaction
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern-recognition receptors
PBP	Penicillin binding protein
PMNs	Polymorphnucleous cells
pg	Pictogram
KCN	Potassium cyanide medium
KD	Kilo Dalton
RSV	Respiratory syncytial virus
ROS	Reactive Oxygen Species
RNIs	Reactive Nitrogen Intermediates
SD	Standard Deviation
SDA	Sabouraud's Dextrose Agar
TLRs	Toll-like receptors
TCA	Tri-chloroacetic acid
UTI	Urinary tract infection
WBCs	White blood cells
Th1	T helper cell 1
Th2	T helper cell 2
TLRs	Toll-like receptors
TCA	Tri-chloroacetic acid
TNF	Tumor Necrosis factor
TM	Tympanic membrane
URT	Upper respiratory tract
URTI	Upper respiratory tract infections
UTI	Urinary Tract Infection
Y	Yates Correction for Continuity

CHAPTER ONE

INTRODUCTION and LITERATURE REVIEW

1. Introduction & Literatures Review

1.1: Introduction

Otitis media is a group of diseases in the middle ear, which can be divided into two major diagnostic groups: acute otitis media (AOM) and otitis media with effusion (OME)(unSdgaard *et al*., 2021).Otitis media (OM) is an inflammation of the middle ear with acute otitis media (AOM) being an acute inflammation of the middle ear(Sharma *et al*., 2018). The diagnosis of AOM should be based on acute symptoms, presence of middle ear effusion , and signs of acute middle ear inflammation with a bulging of the tympanic membrane (TM)(Lieberthal *et al*., 2013) .

OM is one of the most common childhood infections and is a major cause of morbidity in children. The pathogenesis of OM is multifactorial, involving the adaptive and native immune system, Eustachian tube dysfunction, viral and bacterial load, genetic and environmental factors (Rye *et al*., 2012).

The community Health education and the environment play an important role in the spread of pathogens and for the case of inflammation , as the cleaning of the ear canal is incorrect and does not follow the conditions of hygiene and swimming in contaminated water all lead to get infection and the spread of pathogens (Kadhim *et al*., 2018).

OM is a common disease in people all over the world, which may be caused by bacterial , viral , fungal and allergic ,the most

common bacterial pathogens as *Staphylococcus aureus* , *Pseudomonas aeruginosa, Proteus spp.* , *Klebsiella spp.* and *E.coli (*Okesola and Fasina, 2012*).*This disease is an inflammation of middle ear which is related anatomically and pathologically with upper respiratory tract , therefore the nasopharynx is a natural reservoir for several bacterial species including a virulent bacteria and middle ear pathogens of healthy persons, 20- 40 %accompanied infection together (Coleman *et al*. , 2018).

Complications of OM can develop following untreated or incompletely treated infections as well as cases with adequate antimicrobial treatment, and are also seen with infections caused by bacteria that are resistant toantibiotics (Zapalac *et al.* , 2002).

In acute otitis media (AOM) it commonly develops in association with an infection off the upper respiratory tract that extends from the nasopharynx to the middle ear through the Eustachian tube (ET) (Springhouse et al. , 2005).

OME is defined as the presence of fluid in the middle ear with absence of clinical signs or symptoms that related to acute ear infection. Loss of the hear is the main feature of OME (Rovers *et al.* , 2004 ; Rosenfeld *et al.* , 2004) .

Chronic suppurative otitis media (CSOM) is defined as a chronic inflammation of the middle ear and mastoid cavity, which presents with recurrent ear discharges or otorrhoea

through a tympanic membrane perforation. The disease usually begins in childhood (Coker *et al.* ,2010), as a tympanic membrane perforation due to an acute infection of the middle ear, known as acute otitis media (AOM), or as a sequel of less severe forms of otitis media (e.g. secretary OM) (Qureishi *et al.* , 2014) .

Antimicrobial resistance is a global public health challenge, which has accelerated by the overuse of antibiotics worldwide. Increased antimicrobial resistance is the cause of severe infections, complications, longer hospital stays and increased mortality (Llor and Bjerrum ,2014).

The relationship between allergy and otitis media with effusion is caused by mediators of inflammation, cytokines and colony-stimulating factors released by mucosal mast cells and other inflammatory and epithelial cells in the nose and nasopharynx, these mediators produce blockage of the Eustachian tube (ET) through a number of mechanisms, which may include local injury or vascular or neural-mediated changes in the (ET) opening pressure and in middle ear perfusion. It is likely that the nasal allergic response in patients predisposes to (ET) blockage and, if prolonged, causes changes in gas absorption in the middle ear space. This gas exchange primarily involves nitrogen absorption, which may take several days to develop. This persistent under pressure will then lead to middle ear effusion (Schilder *et al.* , 2015).

The early diagnosis of pathological condition will prevent the complications associated with an infection similar occurrence such as brain abscesses, meningitis and thrombus side pockets (Kadhim *et al*. ,2018).

These cytokines trigger acute phase responses and induce proliferation and differentiation of T and B cells (Turner *et al.* , 2014).

Interleukin-10 (IL-10) plays an important role in regulating the inflammatory response by inhibiting the release of immune mediators, phagocytosis, and the presentation of antigens to T-cells and macrophages (Skotnicka and Hassmann ,2008; Sabat *et al*. , 2010).

Cytokines play a central role as initiators, mediators and regulators of middle ear inflammation and subsequent molecular-pathological processes in middle ear tissues, leading to histopathological changes in the middle ear cavity and the pathogenesis of OME (Smirnova *et al*. ,2002).

Aim of the study

. The present study aimed to determine the relationships between the microbial infections and some of immunological parameters for otitis media achievement of this aim were by the following objectives:

1. Isolation and identification of some aerobic bacteria and fungi causes of infection from patients with otitis media.
2. Determination of antibiotic sensitivity test for among bacterial and fungal isolates.
3. Determination of some immune parameters such as human interferon gamma (IFN-γ), human Interleukin 10 (IL-10) and Human Cytotoxic T Lymphocyte Associated Antigen 4 (also known as CTLA-4) concentration that associated with Immune response against microbial infections

1.2 : Literature Review

1.2.1: Historical aspect of otitis media

Several studies have shown that in recent years' incidence of acute otitis media (AOM) has declined worldwide. However, related medical, social, and economic problems for patients, their families, and society remain very high. Better knowledge of potential risk factors for AOM development and more effective preventive interventions, particularly in AOM-prone children, can further reduce disease incidence. However, a more accurate AOM diagnosis seems essential to achieve this goal. Diagnostic uncertainty is common, and to avoid risks related to a disease caused mainly by bacteria, several children without AOM are treated with antibiotics and followed as true AOM cases(Esposito *et al*., 2021)

Despite the immense advancements in medicine since Hippocrates' time, his description of complicated otitis media (OM) does not sound outdated to a modern otorhinolaryngology's. Not only have the knowledge of anatomy and the understanding of the disease's underlying conditions advanced since his time, but fairly recent great developments have been made in the diagnostics, and treatment of diseases. While living in the age of antimicrobials means were often able to prevent the most severe complications that Hippocrates saw, still see patients, young and old, presenting with OM and its complications (Laulajainen-Hongisto, 2016).

Then antimicrobials arrived, with sulfonamides being introduced in the 1930s and penicillin, streptomycin, chloramphenicol, and tetracycline all

beingintroduced in the 1940s (Simpson *et al*., 2013).

Before antibiotics, complications due to OM were difficult to treat, with *Streptococcus pyogenes (StrA)* being the most common pathogen that led to AOM complications (Vergison *et al*.,2008), in less than 100 years since their introduction, however, the effectiveness of antimicrobials has been reduced. Currently, bacterial resistance to antimicrobials is a growing global problem due, in large part, to the often excessive use of antibiotics in the human medical field as well as in agriand aquaculture (Heuer *et al.* , 2009).

Thankfully, tools to aid in the diagnostics and treatment of OM and its complications have been developed. Computed tomography (CT) imaging was introduced in the 1970s (Simpson *et al*.,2013), and magnetic resonance imaging (MRI) was utilized in clinical practice starting in the early1980s (Ai *et al*., 2012).

1.2.2: The anatomy of ear

The ear has three major parts; the outer ear (Theelen et al.), middle ear (ME) and inner ear . The outer ear consists of the pinna (auricula) and the external auditory canal (EAC), at the end of the (EAC) lies the Tympanic membrane (TM), also known as the ear drum which forms the lateral border of the middle ear (Drake *et al*., 2005). The middle ear (ME) is an air chamber which contains three bones; the malleus, incus and stapes. The (ME) is connected to the nasopharynx area through Eustachian tube (ET). The inner ear contains the hearing and balance organs (cochlea) (Barbara and Homeier, 2005).

The middle ear (ME) is an air-filled cavity lined by mucous

membrane, situated inside the temporal bone it consists of the squamous, petrous, mastoid, tympanic, and styloid bones (Laulajainen-Hongisto, 2016) Figure (1-1).

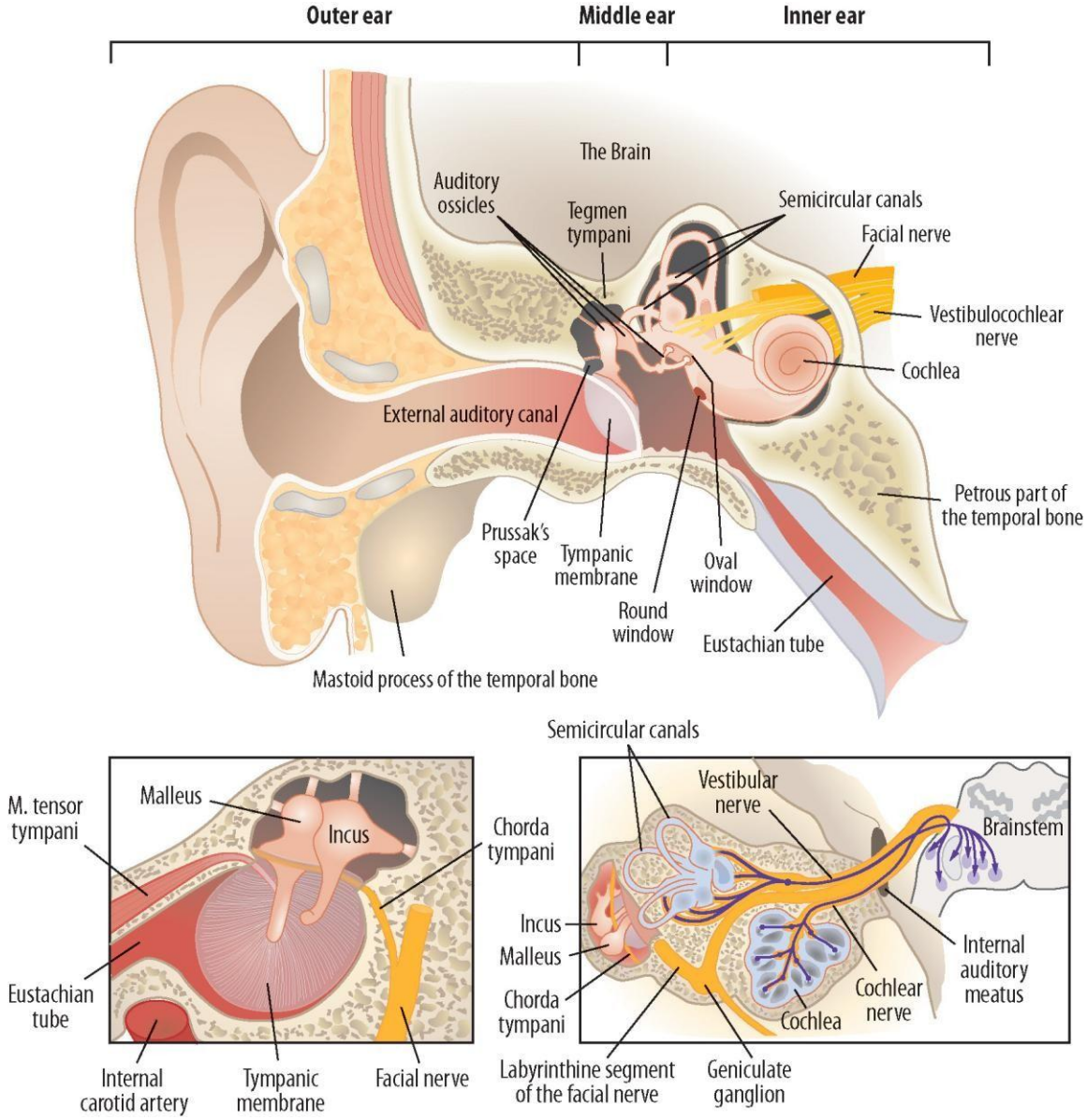

Figure (1-1): Anatomy of The Ear (Laulajainen-Hongisto, 2016)

1.2.3: Otitis media

Otitis media (OM) refers to inflammation of the middle ear. Although several subtypes of otitis media are distinguished, the term is often used synonymously with acute otitis media. It is very common in childhood but may occur at any age (Lieberthal *et al*., 2013).

OM is a major health problem in both developed and developing countries (Hirapure and Pote, 2014). It's an inflammation of the middle ear and mastoid process which could be acute purulent otitis media, otitis media with effusion and chronic suppurative otitis media (Al-Sadeeq *et al.* , 2018).

Tympanic membrane (TM) perforations and bone erosions may allow for the spreading of infection to and from the middle ear, allowing for complications to arise. Complications of OM can be classified intratemporal and extratemporal (either intracranial or extracranial). Anatomical or immunological conditions may predispose patients to these complications. The thinner bony structures of children may more easily be resorbed by infection, allowing for easier development of AOM complications (Stenfeldt *et al*., 2014).

OM is a multifactorial disease; environmental, bacteriological, host, immunological, and genetic factors affect its development (Rye *et al*., 2012).

From twin and family studies, we know that genetic factors play a major part in susceptibility to OM with heritability being

approximately 40-70% (Hafrén *et al.* , 2012 ; Mittal *et al.* , 2014a)

1.2.4: Types of otitis media

1.2.4.1: Acute otitis media

Acute otitis media (AOM) is a common pediatiric infection; disease etiology and pathogenesis are complex and begin with colonization of mucosal surfaces in the upper respiratory tract by AOM bacterial pathogens (Bogaert *et al*., 2004). Several hundred different bacterial taxa can potentially colonize the upper respiratory tract of a single individual (Andersson *et al.* , 2008 ; Nam *et al.*,2011) .

Acute otitis media (AOM) is an infection that is particularly common in children. The bacterial etiology of AOM, in both children and adults, affects its clinical picture. While in some cases the infection can simply be carefully monitored without treatment, antimicrobials are often prescribed. Caution is required, however, when prescribing antimicrobials as their excessive use has led to antimicrobial resistance; this resistance has been seen among some of the causative pathogens for these infections (Laulajainen-Hongisto, 2016).

AOM infection of the middle ear, is one of the most common infections in children, though it affects adults as well. Middle ear infections, and the problems caused by them, however, differ between children and adults. Children are

prone to developing AOM due to anatomical and immunological immaturity, whereas adult ear infections are typically chronic in nature (Bluestone *et al.* , 2008).

. Acute otitis media often develops as a complication of viral upper respiratory tract infections (URTI). Many viruses can cause URTI: rhino-, corona-, adeno-, entero-, and influenza viruses. The respiratory syncytial virus in particular has been associated with AOM (Chonmaitree *et al.*, 2008).

Viruses alone can cause acute middle ear infection, but the interplay of viruses with bacteria is important in the development of AOM, with the resulting AOM being a combination of a viral and bacterial infection (Nokso- Koivisto *et al.* ,2015).

A healthy TM is translucent and pearl gray in color (Figure (1-2) A). Changes in the translucency (from translucent to semi opaque or opaque) or color (from gray to yellow, white, or red) of the TM are used to evaluate patients with AOM, however, a bulging of the TM, in particular, has been found to be an important sign of AOM (Lieberthal *et al.* 2013) (Figure 1-2) B).

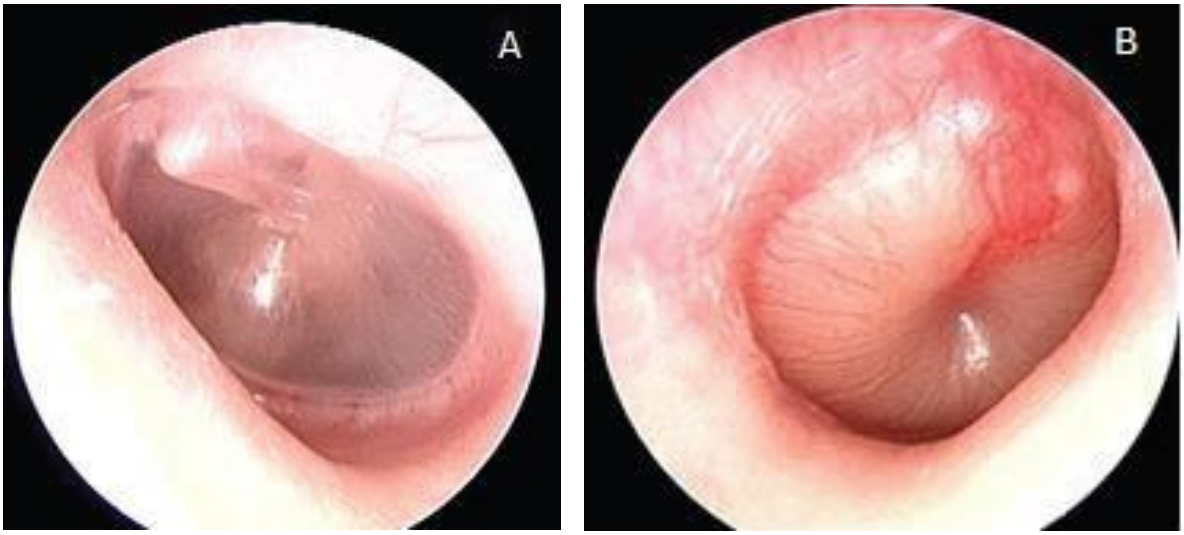

Figure (1-2): The Tympanic Membranes of a Healthy Patient (A) and ofa Patient with Acute Otitis Media(B) (Laulajainen-Hongisto, 2016)

1.2.4.2: Otitis media with effusion

Otitis media with effusion (OME), also or glue ear, is simply collection of fluid that occurs within the middle ear space due to the negative pressure produced by altered Eustachian tube function (Lieberthal *et al.*,2013).

This can occur purely from a viral URTI, with no pain or bacterial infection or it can precede and / or follow acute bacterial otitis media (Rockvill and Bethesde, 2006; Perera *et al.* , 2013).

AOM represents an acute infective (bacterial and/or viral) process, whereas OME is characterized by the presence of a middle ear effusion in the absence of symptoms and signs of

acute inflammation (Kubba *et al*. ,2000). OME and AOM are the leading cause of primary care visits, and the most frequent reason for antibiotics or surgery (Rovers *et al.* ,2004).

AOM is a bacterial infection of the middle ear that causes fever and pain, has a risk of acute complications, and manifests itself as an erythematous, bulging tympanic membrane. If the membrane is perforated, a bloodstained discharge may be present. This AOM differs from that of otitis media with effusion (OME). OME causes temporary hearing loss; in the long run, it is said to cause bone resorption and retraction pockets. However, the patient shows no signs of illness like pain or fever. In general, it is self-limiting and most often it does not leave the patient with a functional hearing impairment (De Ru and Grote , 2004) .

1.2.4.3: Chronic otitis media

Chronic otitis media (COM) with cholesteatoma, a persistent hole in the ear drum sometimes can lead to a growth (tumor) in the middle ear made of skin cells and debris. Cholesteatomas can causes hearing loss and are prone to get infected, which can cause ear drainage (Lieberthal *et al*., 2013).

COM may be suppurative or non suppurative chronic, chronic suppurative otitis media (CSOM) involves a perforation (hole) in the tympanic membrane and active bacterial infection within the middle ear space for several weeks or more. Discharge presents in the active state of

disease, CSOM is a form of OM which is still one of the commonest medical problems, and some studies defined this illness as a persistent disease, this disease in much more common in persons with poor Eustachian tube function. Hearing impairment often accompanies this disease (Leach and Morris, 2006).

Chronic otitis media describes some long-term problems with the middle ear, such as a whole (perforation in the eardrum that does not heal) or a middle ear infection (otitis media) that doesn't improve or keeps returning is characterized by recurrent or persistent ear discharge (otorrhea) over (2-6 weeks), through a perforation of the TM. COM occurs when the ET becomes blocked repeatedly due to allergies, multiple infections, ear trauma or swelling of the adenoids (Brunton and Pichichero , 2005).

Gram negative bacilli, particularly *Pseudomonas aerugenosa* , *Proteus ssp.* and anaerobes were the predominant causative pathogens for this disease (Lee *et al.*, 2002).As in other chronic infections, bacteria involved in CSOM infection may form biofilms that are resistant to treatment (Lampikoski *et al.*, 2012). *Pseudomonas aeruginosa* and *Staphylococcus aureus* are common pathogens involved in CSOM, though *Proteus vulgaris*, *Klebsiella pneumoniae*, *Enterobacteriaceae*, and anaerobes are also found in this group of patients (Brook , 2005 ; Yeo *et al*., 2008 ; Mittal *et al.* , 2015).

1.2.5: Risk factors of otitis media

Acute ear infection, ear infections are the most common reason; the rate of acute otitis media has been raising over the past decades. Ear infections are more likely to occur in the autumn and winter. It is possible to get an ear infection with or without the risk factors list below. A family history of AOM is a risk factor for development of AOM in a child (McCormick *et al.* , 2011).

Ciliary dysfunction, cleft palate, craniofacial anomalies, and down syndrome are additional known risk factors for AOM (Gould and Matz , 2010). The more risk factors you have, the greater your likelihood of getting an ear infection:

1. **Age and sex**

Three-quarters of children will experience an ear infection before their third birthday, and nearly half of these children will have three or more infections by age 3. The risk of ear infections is higher in children because their immune systems have less experience with common viruses than do those of adults. Virus infections likely are the direct or indirect cause of most middle ear infections. Moreover, children's shorter Eustachian tubes (the Eustachian tube is the small channel which lets air pass from the nose into the middle ear) make it easier for bacteria to gain access to the middle ear. The male

more prone than female to otitis media,that males are more susceptible to environmental conditions in their lives such as swimming pools or in other rivers and ponds than females (Jacoby *et al*., 2007).

2. Daycare attendance

Children in daycare or nursery schools are more likely to get ear infections because they are exposed to more upper respiratory infections that can subsequently infect the middle ear. While daycare is a necessary "fact of life"

for many children, it is also one of the strongest risk factors for ear infectionfor the development of OME (Asoegwu *et al.* , 2013).

3. Exposure to cigarette smoke

Children who live with adults who smoke cigarettes are more likely to develop ear infections, a correlation between environmental tobacco smoke (ETS), acute otitis media (AOM) and other adverse etiologic outcomes (Csákányi *et al.* , 2012)

4. Poverty

While ear infections are common in families from all levels of income, they tend to be more frequent and more prolonged in poor children (Andersson*et al.*, 2008).

5. Bottle feeding

Children who are bottle-fed, especially for 4-6 months or longer, have more and longer ear infections than do breast-fed infants (Bogaert *et al.*,2004).

6. Allergies and asthma

People with allergies or asthma adult are more likely to develop ear infections. The reasons for this increased risk remain incompletely understood (Lozupone and Knight ,2008). Children with immune disorders, including HIV and AIDS, and those receiving immunosuppressive therapy are more likely to develop ear infections because their bodies fight bacteria and viruses less effectively (Labout *et al*., 2011).

7. Other infections

Children are more likely to get an ear infection if they have a common cold, pharyngitis/tonsillopharyngitis, or perhaps some eye infections. Although ear infections are not themselves contagious, colds, sore throats, and other respiratory infections are readily passed from person to person (Labout *et al*. ,2011).

1.2.6: Etiological agents

1.2.6.1: Bacterial causes of otitis media

1.2.6.1.1: Gram positive bacteria

1.2.6.1.1.1: *Staphylococcus spp.* and *Staphylococcus aureus*

The genus *Staphylococcus* is composed of Gram-positive bacteria with diameters of 0.5-1.5 μm, characterized by individual cocci that divide in more than one plane to form grape-like clusters. These bacteria are non-motile, no spore

forming facultative anaerobes, featuring a complex nutritional requirement for growth (Costa *et al*., 2013). However, in general they require an organic source of nitrogen, supplied by 5 to 12 essential amino acids, e.g. arginine, valine, and B vitamins, including thiamine and nicotinamide (Harris *et al*., 2002).

The optimum growth temperature is 30°C-37°C.They have a fermentative metabolism. *Staphylococcus* species are usually catalase positive and are also oxidase negative with the exception of the *S. sciuri* group (*S. sciuri, S. lentus* and *S. vitulinus*), *S. fleuretti* and the *Macrococcus* group to which *S. caseolyticus* has been assigned (Vernozy-Rozand *et al.*, 2000).

Staphylococci are tolerant to high concentrations of salt, most strains can grow in the presence of 9% sodium chloride and show resistance to heat. Pathogenic staphylococci are commonly identified by their ability to produce coagulase, and thus clot blood (Harris *et al.*, 2002).

S. aureus is gram positive cocci accruing in grape formation, aerobic or facultative anaerobic, catalase positive (Garrity *et al*., 2005).

S. aureus is one of the main causes of hospital- and community-acquired infections which can result in serious consequences (Diekema *et al*., 2001).

Infections by *Staphylococcus* usually result from a combination of bacterial virulence factors and diminution in

host defense. Important microbial factors include the ability of the staphylococci to survive under harsh conditions aided by its cell wall constituents and the production of enzymes and toxin, that promote tissue invasion and enhance its capacity to persist intracellular within phagocytes, *Staphylococcus* expresses many potential virulence factors that play important role in pathogenesis (Al-Zuhairi *et al*., 2005).

1.2.6.1.1.2 : *Staphylococcus epidermidis*

Staphylococcus epidermidis is a very hardy microorganism, consisting of non-motile, Gram-positive cocci, arranged in grape-like clusters. It forms white, raised, cohesive colonies about 1–2 mm in diameter after overnight incubation (Salyers and Whitt, 2002).

This bacterium is facultative anaerobe, which are best produced aerobically. Temperature for growth range 10-42ºC, optimum temperature 37ºC and optimum pH 7.5 (Cheesbrough, 2006).

This species is coagulase negative, catalase positive, oxidase negative, weakly Dnase positive, urease positive, novobiocin (5µ gm) sensitive, Polymyxin B resistant, ferment sucrose, D-manose and maltose but not D- mannitol, D-trehalose, D-xylose and D-cellobiose, do not form acid but produce acetoin and phosphatase and reduce nitrate (Cheesbrough, 2006; Bannerman, 2007).

1.2.6.1.1.3: *Streptococcus pyogenes*

Strepto*coccus pyogenes*, (colloquially named "group A streptococcus" (GAS), is a Gram-positive bacterial pathogen that can cause both non-invasive and invasive disease (iGAS), as well as nonsuppurative sequelae. This includes pharyngitis, scarlet fever, impetigo, cellulitis, type II necrotizing fasciitis, streptococcal toxic shock syndrome, acute rheumatic fever and post-streptococcal glomerulonephritis(Avire *et al*., 2021).

streptococcus pyogenes is a major human-specific bacterial pathogen that causes a wide array of manifestations ranging from mild localized infections to life-threatening invasive infections. Ineffective treatment of *S. pyogenes* infections can result in the post infectious sequela acute rheumatic fever and post-streptococcal glomerulonephritis. Moreover, it causes invasive infections like necrotizing fasciitis and toxic shock syndrome that is associated with and high morbidity and mortality. Streptococci are gram-positive, catalase-negative, coagulase-negative cocci that occur in pairs or chains. They are divided into three groups by the type of hemolysis on blood agar: beta-hemolytic (complete lysis of red cells), a hemolytic (green hemolysis), and gamma-hemolytic (no hemolysis). Beta-hemolytic streptococci are characterized as group A streptococci (*Streptococcus pyogenes*) and group B streptococci (*Streptococcus agalactiae*)(Kanwal and Vaitla, 2020).

1.2.6.1.2: Gram negative bacteria

1.2.6.1.2.1: *Pseudomonas*

Most *Pseudomonas* known to cause disease in human are associated with opportunistic infections especially *Pseudomonas aeruginosa*. It is strict aerobic but it grows in the absence of O_2 if NO_3 is available as a respiratory electron acceptor. *Pseudomonas aeruginosa* has very simple nutritional requirements, sometimes producing a sweet or grape like or corn taco like odder (Ray *et al.* , 2015).

The high motility associated with this infection is due to a combination of weakened host defense, bacterial resistance to antibiotics and the production of extracellular bacterial enzymes and toxins (Anzai *et al*., 2000).

. The pathogenesis of *Pseudomonas* infections is multifunctional as suggested by the number and wide array of virulence determinants possessed by the bacterium (Miyata *et al*., 2003).

Multiple and divers determinants of virulence are expected in the wide range of disease caused, which include septicemia, urinary tract infection, pneumonial, otitis media, chronic lung infection, endocarditis, dermatitis, osteochondritis bone and joint infections, gastrointestinal infection, eye infection, Central Nervous System (CNS) infection, skin and soft tissue infection, the ultimate *Pseudomonas* infection may be seen as composed of three distinct stages:

1. Bacterial attachment and colonization
2. Local invasion
3. Disseminated systemic disease. The disease process may stop at any stage (Prithiviraj *et al*.,2005).

1.2.6.1.2.2 : *Escherichia coli*

One of the most important Enterobacteriaceae species is gram negative rods, usually motile, produce polysaccharides capsule, positive tests for indole, lysine decarboxylase and mannitol fermentation, and produces gas from glucose (Brook *et al*.,2004).

E. coli causes UTI, chronic OM and sinusitis (Ad-Dahhan *et al*., 2005).

Typical morphology with an iridescent sheen on differential media such as Eosin Methylene Blue (EMB) agar, and the grow on non-selective media, most strain ferment lactose producing large red colony on MacConkey agar and this bacteria is predominant among aerobic commensal bacteria in healthy human intestine (Rivas *et al.* , 2015).

1.2.6.1.2.3 : *Proteus*

It is a small gram negative, oxidase negative, urease positive which grows on potassium cyanide medium (KCN), and not ferment lactose on MacConkey agar, *Proteus* moves very actively by means of peritrichous flagella, resulting in swarming on solid media (*Proteus* produce swarmer cells which are elongated forms created when cells fail to septate or

divide, these cells, which are profusely covered with flagella, act in concert to produce swarming motility on solid media) (Brook *et al*., 2004).

Proteus spp. is common cause of chronic OM (Madana *et al*. , 2011). This bacteria have many virulence factors like fimbriae, flagella, outer membrane proteins, capsule antigen, lipopolysaccharide and IgA protease, which attribute to the pathogenicity of this bacteria (O'Hara *et al*., 2000; Green Wood *et al*., 2002).

1.2.6.1.2.4 : *Klebsiella pneumoniae*

It was a gram- negative, non-motile, encapsulated found in the normal flora of the mouth, skin and intestine (Ray *et al.* , 2015). It is characterized by polysaccharide capsule (which give their colonies their characteristic mucoid appearance), rod-shaped, facultative anaerobic. It is non-spore forming, flagellated, produces large sticky colonies when plated on nutrient media (Forbes *et al.* ,2016).

It is clinically the most important member of *Klebsiella* genus of Enterobacteriaceae. *Klebsiella spp.* is one of the major pathognes responsible for nosocomial infection. Colonization of the respiratory tract by *Klebsiella pneumoniae* is very common in hospital patients receiving antibiotics. *Klebsiella* infections are encountered more often now than in past. This is probably due to bacterium antibiotic properties (Green wood *et al*., 2002).

Klebsiella pathogenicity may be attributed to its production

of heat stable enterotoxin. The major virulence factor of which plays an essential role in pathogenesis is thick capsule (it's antiphagocytic) and fimbriae (pili) (Podschun *et al*., 2001).

1.2.6.1.2.5 : *Acinetobacter*

Acinetobacter is a genus of Gram negative bacteria; it belongs to the family of *Moraxellaceae*, non-motile, non-pigment, oxidase negative nitrate negative. *Acinetobacter spp.* is strictly aerobic, non-fermentation of lactose (Visca *et al.* , 2011).

It consists of ubiquitous Gram negative bacilli that initially encompassed a heterogeneous collection of non-pigmented, oxidase positive or negative, Gram negative rods (Dijkshoorn and Nemec, 2008).

It currently comprises Gram negative coccobacilli that are non-motile, non-fermentative, non-spore forming, strictly aerobic, catalase positive (Vila and Pachón, 2008).

1.2.6.1.2.6 : Other Gram negative bacteria genus

Citrobacter spp. share all the general properties and biochemical characteristics of the family *Enterobacteriaceae*, including the following: Gram-negative rod, catalase-positive and oxidase-negative, growth on MacConkey agar, reduction of nitrate to nitrite, growth both aerobically and anaerobically, and fermentation of glucose and other carbohydrates. Most isolates are motile and utilize citrate as a sole carbon source, but lack urease and lysine decarboxylase

activity; production of hydrogen sulfide is variable, occurring with *C. freundii* and a few other species (Long *et al.* , 2012) .

1.2.6.2: Fungal causes of otitis media

Fungi can either be the primary pathogen or be superimposed on bacterial infections or can be secondary pathogen in previously perforated tympanic membrane. It is mainly characterized by pruritus, otalgia, aural fullness, hearing impairment and tinnitus. Various predisposing factors have been proposed for fungal ear infection, including immunocompromised host, steroid usage, trauma, swimming, ear picking, use of headwear, use of oils, instrumentation of ear, fungal infection elsewhere in the body like dermatomycosis and malnutrition in children (Ray *et al.* , 2015)

1.2.6.2.1: *Aspergillus niger*

Aspergillus niger is a filamentous ascomycete fungus that is ubiquitous in the environment and has been implicated in opportunistic infections of humans. In addition to its role as an opportunistic human pathogen, *A. niger* is economically important as a fermentation organism used for the production of citric acid. Industrial citric acid production by *A. niger* represents one of the most efficient, highest yield bioprocesses in use currently by industry(Baker *et al.* , 2006).

Aspergillus niger is a haploid filamentous fungus which is used for waste management and biotransformation's in addition

to its industrial uses, such as production of citric acid and extracellular enzymes. It is most commonly found in decaying vegetation, soil, or plants, but it cannot be considered particularly dangerous in comparison to *Aspergillus fumigatus*, which is the most prevalent airborne pathogen. *Aspergillus niger*, the most abundant mold found in the environment, has also been the source of several bioactive compounds and industrial enzymes (Schuster *et al*., 2002). Wide spectrum of fungal agents such as *Aspergillus, Penicillium, Mucor, Rhizopus, Scopulariopsis, Absidiaand* and *Candida* are involved, species of *Aspergillus* and *Candida* being the most common etiological agents. *Aspergillus spp.* and *Candida spp.* as the predominant fungi (Ray *et al.* , 2015; Mohamad *et al.* , 2017).

1.2.6.2.2: *Candida Species*

The traditional criterion for distinguishing between different forms is cell shape. Hyphae that develop from an unbudded yeast cell (also termed a blastospore) have no constriction at the neck of the mother cell and have parallel sides along their entire length. The formation of unconstricted filaments in response to serum is the basis of the ‘germ tube test’, which is used in clinical diagnoses to distinguish *C. albicans* from other *Candida* species(Sudbery *et al*., 2004).

The *Candida parapsilosis* family has emerged as a major opportunistic and nosocomial pathogen. It causes multifaceted pathology in immuno-compromised and normal hosts, notably low birth weight neonates. Its emergence may relate to an ability to colonize the skin, proliferate in glucose-containing solutions, and adhere to plastic. When clusters appear, determination of genetic relatedness among strains and identification of a common source are important. Its virulence appears associated with a capacity to produce biofilm and production of phospholipase and aspartyl protease. Further investigations of the host-pathogen interactions are needed(van Asbeck *et al*., 2009).

1.2.6.2.3: *Malassezia furfur*

Malassezia furfur is a yeast species belonging to Malasseziomycetes, Ustilaginomycotina and Basidiomycota that is found on healthy warm-blooded animal skin, but also involved in various skin disorders like seborrheic dermatitis/dandruff and pityriasis versicolor. Moreover, *Malassezia* are associated with bloodstream infections, Crohn's disease and pancreatic carcinoma(Theelen *et al.,* 2021).

Malassezia species are not only commensals and pathogens to humans, but also inhabit all warm-blooded animals, with some species found exclusively on animals(Theelen *et al*., 2018; Bond *et al*., 2020).

1.2.6.3: Viral Infection

Otitis media is always preceded by viral infection of the nasopharyngeal and Eustachian tube epithelium: the ‘common cold’ or viral URTI (Nokso-Koivisto *et al.*, 2015). Bacterial otopathogens that are colonized in the nasopharynx do not cause any harm until the virus initiates the inflammatory process in the nasopharynx. A wide variety of viruses that cause URTI symptoms can induce AOM development. These include the following viruses in the order of importance: respiratory syncytial virus (RSV), rhinovirus, adenovirus, coronavirus, bocavirus, influenza virus, parainfluenza virus, enterovirus and human metapneumovirus (Chonmaitree 2008; Nokso-Koivisto *et al.,* 2015).

1.2.7: Antimicrobial agents

Although antibiotics are the most preferred and prescribed drugs in incidents of OM, the antimicrobial resistance still remains a persistent among bacterial pathogens of otitis media (Jonathan *et al.* , 2016).

Medical treatment of OM is an important step in achieving a drug ear (Segal *et al*. ,2005), and it includes either aural toilet or antimicrobial agents, and the later (antimicrobial agents) may be used either topically or systemically or by both routs. Topical treatment may include antiseptic or antimicrobial agents which have been extensively used in the treatment of

adults (Rosenfeld *et al*., 2006), some studies showed that optical use of a mixture containing neomycin and polymyxin B may contribute to sensor neural hearing loss in patients with chronic OM (Corti and Imhof , 2008).

Amoxicillin, Augmentin (Amoxicillin + Clavulanic acid) and second generation of Cephalosporin were the most common systemic antibiotic used for OM treatment (Coker *et al*., 2010).

Ear wax is composed mostly of dead skin cells and keratin with a small mixture of cerumen, sweat and oil. Cerumen is secreted from the ceruminous glands located in first third outer part of the ear canal and is thought to be composed mainly of cholesterol, squalene, wax ester, ceramides and triglycerides. Ear wax shows that some of the antimicrobial properties of the cerumen can be allributed to presence of antimicrobial peptides, human Beta- defensing 1 and human Beta-defensing 2. Other resent studies have shown that cerumen directly inhibits the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*, but its effect on the growth of *E. coli* remains be determined. In the absence of cerumen, the ear canal would be an optimal environment for microbes (Joo Yoon *et al*., 2008).

1.2.8: Immune response associated with otitis media

Protection from otitis media (OM) due to bacterial and viral pathogens depends on innate and pathogen-specific adaptive immunity. OM-prone children may also be unusually vulnerable to other respiratory infections, such as viral upper respiratory infections, sinusitis, and pneumonia. This broad susceptibility suggests that OM-prone children may have selective immunologic derangements or deficits predisposing development of an OM-prone phenotype. Effective active immunoprophylaxis for OM will be possible only when immunologic mechanisms for predisposition are more fully understood(Kyd *et al*., 2017). Inflammatory responses are strictly regulated by coordination of pro-inflammatory and anti-inflammatory mediators. Interleukin-10 (IL-10) have typically the biologic anti-inflammatory effects on monocytes, but uncertain effects on polymorphonuclear leukocytes (PMNs). The PMNs are the first line of cellular response for host defense during acute inflammation (Lee *et al.* , 2002).Host immune responses to viral and bacterial infections were reported to be closely associated with complete recovery from OM, as well as with the persistence and recurrence of this disease, innate immunity is already functioning at birth, whereas adaptive immunity develops as pathogens are encountered (Qureishi *et al*. ,2014 ; Mittal *et al.* ,2014a).

Susceptibility to OM may, in adults, be affected by how

well an individual's (innate) immunity functions (Mittal *et al.*, 2014b).

Innate and adaptive immune responses within the middle ear cavity of patients with various types of OM have therefore been evaluated, including assessments of cytokine secretion (Mittal *et al.* , 2014a ; Smirnova *et al.* , 2004).

Inflammatory reactions induced by pathogens are regarded as important in understanding the mechanisms of immune response in the middle ear and in treating these patients (Yeo *et al.* ,2008; Ilia *et al.*, 2008).

Inflammation in the middle ear mucosa, which can be provoked by different primary factors such as bacterial and viral infection, local allergic reactions and reflux, is the crucial event in the pathogenesis of otitis media with effusion (OME). Unresolved acute inflammatory responses or defective immunoregulation of middle inflammation can promote chronic inflammatory processes and stimulate the chronic condition of OME **(**Smirnova *et al.* ,2004). The first step in the activation of the human defense mechanism against microbes is the recognition of pathogens by macrophages. Macrophages recognize pathogen-associated molecular patterns (PAMPs), generating intracellular signals and producing cytokines and chemokines, leading to the activation of the acquired immune system (Lee *et al.*, 2013). The recognition and reaction of PAMPs is controlled by pattern-recognition

receptors (PRRs), including Toll-like receptors (TLRs), which bind to infecting microbes and directly induce innate host defense responses (Kim *et al*. , 2010).

The common cold and otitis media are two such disease states, and much has been learned about the various effects of cytokines in each disease. Otitis media, sinusitis, bronchiolitis, pneumonia, and asthma exacerbation are commonly accepted as complications of viral upper respiratory tract infections (Wine and Alper ,2012).

Although the immunologic aetiology and mechanisms of recurrent otitis media have been thoroughly investigated, but the TLRs, cytokines, and no (nitric oxide) were evaluated separately. Little is known about how the innate immune system first reacts with pathogens invading the middle ear cavity, or about the combined expression of TLRs, cytokines, and NO during OME (Lee *et al.* , 2013) .

Pyogenic cocci bacteria like *Staphylococcus aureus* and encapsulated *Streptococcus pneumoniae* are able to resist body defenses by escaping phagocytosis, releasing enzymes and toxins. That will lead to white blood cells (W.B.Cs) death and pus forming due to colonization of pathogen. Any defect in body immune system can cause recurrent suppurative OM infections that are usually called COM infections (Al-Hamadany *et al*.,2013).

(W.B.Cs) death and pus forming due to colonization of

pathogen. Any defect in body immune system can cause recurrent suppurative OM infections that are usually called COM infections (Al-Hamadany *et al*. ,2013).

The composition of the inflammatory substrate in the effusions of allergic otitis media is similar to the late-phase allergic response seen elsewhere in the respiratory tract, such as in asthma and in allergic rhinitis (Nguyen *et al. ,2004).*

1.2.8.1: Human interferon gamma (IFN-γ)

Interferon (IFN)-λ1 is a newly described cytokine, member of type III interferons family, which is known for its antiviral, anti-proliferative and antitumor activity. Recent studies indicated that this cytokine has also immune-regulatory function, but its role in the pathogenesis of autoimmune diseases is not established yet(Dantas *et al*., 2015).

The human immune system is evolved to eradicate or contain any pathogenic challenge and eliminate self-altered cancerous cells. In this regard, IFN-γ has a critical role in recognizing and eliminating pathogens. IFN-γ, being the central effector of cell mediated immunity, can coordinate a plethora of anti-microbial functions. It can serve to amplify antigen presentation through antigen presenting cells (APCs) by enhancing antigen recognition via cognate T-cell interaction, increase the production of Reactive Oxygen Species (ROS) and Reactive Nitrogen Intermediates (RNIs) and induce anti-viral responses (Schroder *et al*., 2004).

Additionally, cancer cells are destroyed by IFN-γ activity via induction of an anti-proliferative state. Immunity to several pathogens is mainly governed by IFN-γ activity. For example, the role of IFN-γ in endowing protection against Chlamydial infections is quite immense (Rothfuchs *et al.*, 2004). Enhanced anti-bacterial and immune protective effects simultaneous with pro-inflammatory responses leading to protection of epithelial monolayers from pathogen mediated injury are also conferred by IFN-γ during Staphylococcus aureus infections (Beekhuizen and van de Gevel, 2007). Release of IFN-γ by the CD4+ helper T cell population contributes to necessary effector cell activation and the generation of antibody mediated responses to *Chlamydia* infections (Naglak *et al*., 2016). The presence of IFN-γ is of absolute necessity in combating mycobacterial infections through its ability to regulate various protective functions and sustain both CD4+ and CD8+ cell activity(Green *et al*., 2013) .

The protective benefits of IFN-γ can also been seen in the context of viral infections, as enhanced survival of neurons infected with varicella zoster virus is observed post IFN-γ treatment(Baird *et al*., 2015). Natural Killer cell -mediated IFN-γ production can successfully limit Hepatitis C virus proliferation in HIV+ (Human Immunodeficiency Virus) patients (Kokordelis *et al*., 2014). Thus, IFN-γ is a robust protective effector molecule mediating protection against a

wide array of pathogenic entities(Kak *et al*., 2018).

1.2.8.2: Human Interleukin 10 (IL-10)

Interleukin 10 (IL-10) is a pleiotropic cytokine with important immune regulation function, not only participating in the differentiation of B cells, but also boosting B cell antibody production in mammals (Wu et al., 2021). Th2 cells are producing cytokines such as (IL-4, IL-5, IL-6 and IL-10) very good helpers for B-cells and would seem to be adapted for defense against bacteria and other extracellular pathogens (Delves et al., 2017).

The immune system is to eradicate infection, but if left uncontrolled it can cause disease. Interleukin-10 (IL-10) is predominantly an anti-inflammatory cytokine made by most cells of the immune system. By limiting immune responses during infection, allergy, and autoimmunity, IL-10 plays a central role in the prevention of immune-mediated damage to the host. However, too much of this suppressive activity of IL-10 can contribute to chronic infections(Howes *et al*., 2014).

IL-10 regulates acute inflammatory reactions and promotes resolution of inflammation, any imbalance in production of these cytokines will induce chronic inflammatory processes (cell-mediated or humoral, or both) or stimulate uncontrolled inflammatory-related damage of the middle ear tissues. IL-10 can be considered as the key cytokine mediators, regulating

switching of the acute phase of middle ear inflammation in the chronic stage, which will lead to the chronic condition of OME (Smirnova *et al.* , 2004).

1.2.8.3: Human Cytotoxic T Lymphocyte Associated Antigen 4 (CTLA-4)

CTLA-4 is an immune checkpoint molecule, primarily found on the surface of T cells (Hosseini et al., 2020). Regulatory T Cells. Regulation of immune responses associated with regulatory T cells (Tregs) diminishes immune responses to microbial infection and normal protective inflammatory responses and may contribute to the chronicity of infection. The presence and role of Tregs in the adenoids of children has been speculated as a contributing factor to COM(Kyd et al., 2017). Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is a critical immunoregulatory molecule (expressed on activated T cells and a subset of regulatory T cells) capable of down-regulating T cell activation(Phan *et al*., 2003).

CHAPTER TWO

MATERIALS and METHODS

2: Materials and Methods

2.1: Materials

2.1.1: Laboratory instruments and equipment:

The main scientific apparatus and technical Instrument with disposable material were listed that used in in Table (2-1).

Table (2-1): Laboratory instrument and apparatus

NO.	Instruments	Company	Country origin
1	Autoclave	Tripod	UK
2	Bench centrifuge	Memmert	Germany
3	Burner	Amal	Turkey
4	Cane tube	Bausch and lomb	USA
5	ELISA system	Biotech	USA
6	Eppendorf tubes	Eppendorf	Germany
8	Hood	Bio LAB	Korea
9	Incubator	Selecta	Spain
10	Light microscope	Olympus	Japan
11	Micropipettes size (5-50 µl, 100-1000 µl , 0.5 – 10 µl)	Eppendorf	Germany
12	Oven	Olympus	Japan
14	Petri dish	Sterilin	England

15	Thermo cycle PCR	Techne (UK)	Ukraine
16	Plain tubes	DMD-DISPO	Syria
18	Refrigerator	Kiriazi	Egypt
20	Slide	Sail Brand	China
21	Sterile hypodermic syringe	EL-dawlia ico	Egypt
22	Sterilize Swab	ATACO	Brand
23	Transfer swab	Al hanof factory	Jorden
24	Vitek 2 system	Biomerieux	France
25	Vortex mixer	Griffin	Germany
26	Gel electrophoresis	Bioneer	Korea
27	PCR system	Clever	USA

2.1.2: Chemical materials

The chemical materials, reagents, stains and solutions used in this study were listed in Table (2-2).

Table (2-2): Chemical materials

NO.	Type of chemical	Company/origin
1	Ethanol 99%	Merck-England
2	Glycerol ($C_3H_8O_3$)	Merck-England
3	Gram stain set	BDH, England

4	Sterile urea, α-Naphthol, KOH, Kovac's reagent	Sigma, USA

2.1.3: Culture media

Culture media were used in the present study are illustrated in Table (2-3): -

Table (2-3): Culture media that used in this study

NO.	Type of media	Manufacturing company	Origin
1	Blood agar base	Himedia	India
2	MacConkey agar	Himedia	India
3	Mannitol salt agar	Himedia	India
4	Eosin methylene blue	Himedia	India
5	Brain heart infusion broth	Himedia	India
6	Sabroud agar	Himedia	India
7	MR_VP broth	Himedia	India
8	Muller Hinton agar	Himedia	India
11	Peptone water	Himedia	India
12	Simmon citrate agar	Diffco-Michigan	**USA**

2.1.4: Antibiotic disks

The Antibiotic discs used in this study were listed table (2-4):

NO.	Antibiotics	Symbol	mg/disk	Manufacturing company
1	Ceftriaxone	CRO	30	Condalab
2	Amikacin	AK	10	Condalab
3	Ciprofloxacin	CIP	5	Condalab
4	Vancomycin	VA	30	Condalab
5	Erythromycin	E	30	Condalab
6	Gentamicin	CN	10	Condalab
7	AMC	AMC	30	Condalab
8	Doxycyclin	DO	30	Condalab
9	Cefepime	CPM	30	Condalab

2.1.5: Antifungal group

The antifungal powder and in the study were listed in table (2-5).

NO.	**Antifungal**	**Type**	**Potency**	**Company/ origin**
1.	Cycloheximide ($C_{15}H_{23}NO_4$)	powder	50 mg in 1 ml of ethanol	CDH ,India

2.1.6: Primer Pairs

The primer pair (Macrogen/Korea) Table(2-6).

Table (2-6): Primer Sequencing and PCR Conditions

Primer	Sequence 5 to 3	Product	Conditions	Referenes
Stap16S -F	CCTATAAGACTGGGATAACTTCGGG	791	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 56 ºC, 30 sec. Step 4: 72ºC, 70 sec. Step 6: 72ºC, 5 min. Step 7: 4ºC, forever	Al-Khafaji*et al*., 2019
Stap16S –R	CTTTGAGTTTCAACCTTGCGGTG			
27F	AGAGTTTGATCCTGGCTCA	618	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 53ºC, 30 sec. Step 4: 72ºC, 150 sec. Step 6: 72ºC, 5 min. Step 7: 4ºC, forever	Loy et al., 2002
1492R	GGTTACCTTGTTACGACTT			

2.1.7: Commercial kits

Commercial Kits were used in the present study are illustrated in Table (2-7):

Table (2-7): Commercial kits that used in this study

NO.	Type of Kit	Company	Country
1	IL10	Bio-Techne China	USA
2	IFN γ	Bio-Techne China	USA
3	CTLA-4	BT- lab	china
4	DNA extraction Kit	Favorgen	Taiwan
5	DNA ladder	Bioner	Korea
6	Green master mix	Bioner	Korea
7	Primers	Bioner	Korea
8	Vitek 2 system kit	Biomerieux	France

2.2: Methods

2.2.1: Reagents and solutions

2.2.1.1: Catalase reagent

The reagent was prepared by adding 3% of H_2O_2 to 100 ml of distilled water, and then stored in a dark bottle. The reagent was used to recognize bacterial capability to produce catalase enzyme (Forbes *et al*., 2007).

2.2.1.2: Oxidase reagent

The reagent was prepared directly by dissolving 0.1 g of tetra methyl-ρ-paraphenylene diamine di hydrochloride in 10 ml of distilled water, and then stored in a dark bottle. The reagent has been freshly prepared, the reagent was used to recognize bacterial capability to produce Oxidase enzyme (Forbes *et al*., 2007).

2.2.1.3: Methyl red reagent

The reagent was prepared by dissolving 0.1g of methyl red in 300 ml of 99% ethanol and then, the volume was completed to 500 ml by distilled water. This reagent was used to identify the complete glucose hydrolysis (MacFaddin, 2000).

2.2.1.4: Kovac´s reagent

It was prepared by dissolving 5g of (P-dimethyl aminebenzaldehyde) in 75 ml of amyl alcohol and then 25 ml of concentrated HCl was added. The reagent used for the detection of indole (MacFaddin, 2000).

2.2.1.5: Gram stain solution

Gram Stain solution was supplied from Syrbio company. The solution was used to study Gram positive and Gram negative bacterial cells, morphology and their arrangement (Forbes *et al.*, 2007).

2.2.2: Preparation of culture media

The culture media were prepared according to the manufacture company and sterilized by autoclave at 121ċ and 15 min (Macfadden, 2000).

2.2.2.1: Muller-Hinton agar

Muller-Hinton agar medium was ready for conferring to the manufacturing company and it was used in antimicrobial susceptibility testing (Forbes *et al*., 2007).

2.2.2.2: MacConkey agar medium

It was used for the primary isolation of most Gram-negative bacteria and to differentiate lactose fermenters from non-lactose fermenters (Winn *et al*., 2006).

2.2.2.3: Brain heart infusion broth

It used for activation the bacterial isolates (Forbes *et al*.,2007)

2.2.2.4: Blood agar medium

Blood agar medium was prepared according to manufacturer instructions by dissolving 40 g of blood agar base in 1000 ml D.W. The medium was autoclaved at 121 ºC for 15 min and pressure 15 pounds per square (psi), cooled to 50 ºC and 5% of fresh human blood was added. This medium was used as enrichment medium for the cultivation of the bacterial isolates and to determine their ability of blood hemolysis (Forbes *et al*., 2007).

2.2.2.5: Peptone water medium

This medium was prepared by dissolving 8 g peptone in 1000 ml of distilled water, the medium was autoclaved at 121 ºC for 15 min and pressure 15 pounds per square(psi), then distributed into test tubes. It was used for the demonstration of the bacterial ability to decompose the amino acid tryptophan to indole (MacFaddin, 2000).

2.2.2.6: Methyl red – vogas-proskauer medium (MR-VP)

MR-VP medium was prepared and used to detect the partial and complete hydrolysis of glucose (MacFaddin, 2000).

2.2.2.7: Brain heart infusion broth with 5% glycerol

This medium was prepared by adding 5 ml of glycerol to 95 ml of BHI broth before autoclaving at 121 ºC for 15 min and pressure 15 pounds per square (psi). The medium was used in preservation of bacteria (MacFadden, 2000).

2.2.2.8: Simmon's citrate medium

Simmon's citrate medium was used for determining the ability of bacteria to utilize citrate as the sole carbon source (MacFaddin, 2000).

2.2.2.9: Mannitol salt agar

This medium was prepared according to the manufacture company. It was used as a selective medium for diffrenation and isolation of *Staphylococcus* species contain 7.5-10 Nacl, selective for *Staphylococcus* and *Micrococcus and* differential for *Staphylococcus* (Macfaddin, 2000).

2.2.3: Subjects of the study

Study population were divided into two groups (otitis media patients group and apparently healthy control group**.**

2.2.3.1: Patients Group

The patient group included 90 patients with otitis media, collected from both sexes (male 57 and female 33) with age range (1-80 years). they were suffering from otitis media in ENT Department of AL-Hilla Teaching Hospital and Imam Sadiq Hospital in Babylon city after clinically diagnosed by consultant physician during the period between October 2021 to February 2022.

2.2.3.2: Exclusion Criteria

Exclusion criteria of otitis media patients were involved:

a. Patients with antibiotics treatment.

b. Patients with disease except otitis media.

2.2.3.3: Ethical Approval:

1-The study was done and the cases were collected after getting the agreement of the patients (verbal consent).

2- Approval of Babylon Science Collage Ethical committee.

3- Before starting the study, permission was taken from Babylon health directorate.

2.2.3.4: Control Group

Thirty (30) healthy as control samples which were apparently healthy control with patients age matched groups and divided into each 15 male and 15 female.

2.2.3.5: Questionnaire Chart

The sheet was carried out for all patients included number, age of patients, and treatment intake.

2.2.3.6: Inclusion Criteria

Inclusion criteria of patients group were depending on clinical diagnosis with otitis media. Patients were diagnosed by a specialized physician depending on clinical finding.

2.2.3.7: Specimen Collection

The blood samples taken from each patient and smears were taken from infected area by sterilized cotton swab, and then samples had been inoculated on different bacterial culture media (MacConkey, Blood, Chocolate and Mannitol Salt Agar) incubated aerobically and anaerobically at 37oC for 24-48 hrs. while (Sabouraud's Dextrose Agar) was used for fungal at 25C° for 5-7 days.

2.2.3.8: Study design

A case control study was conducted during this Figure (2-1).

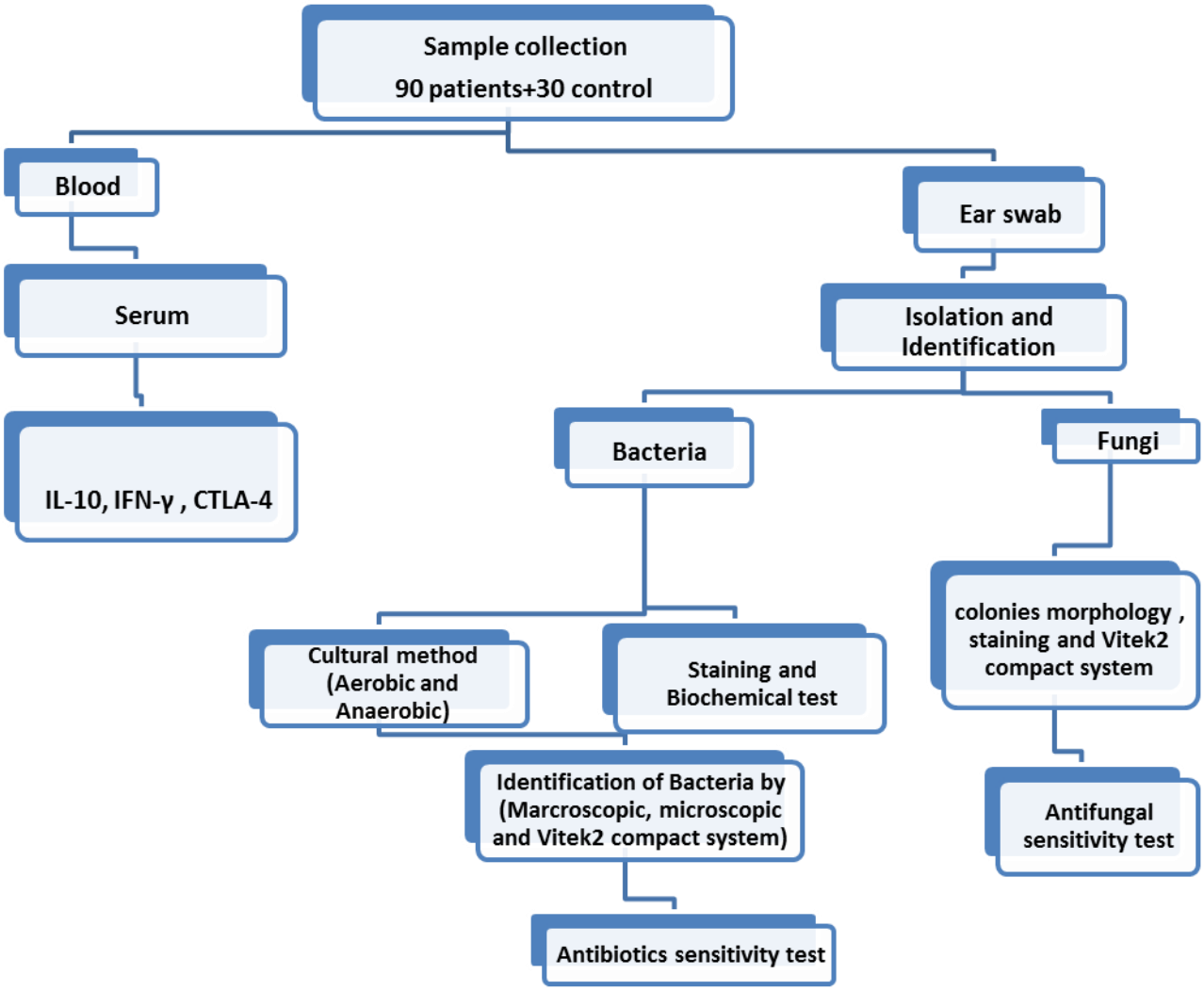

Figure (2-1): The steps study

2.2.3.9: Isolation and identification of bacteriaand fungi

A single colony was taken from each positive culture, negative culture and its identification depended on the morphology properties (colony size, shape, color and nature of pigments, translucency, edge, elevation and texture). Then, colonies were stained by gram stain to observe a specific shape, type of reaction, aggregation and specific intracellular compounds. (Winn *et al*., 2006).

2.2.4: Biochemical tests

2.2.4.1: Catalase test

This test used to detect the ability of bacteria to produce catalase enzyme, after nutrient agar media streaking with bacterial isolate and incubated for 24 hours at 37ºC and then used wooden stick to transfer the bacterial growth and put on the surface of clean slide and then added one drop of 3% (H_2O_2), if result show formation bubbles this indicator to positive, (Forbes *et al*., 2007)**.**

2.2.4.2: Oxidase test

The test depends on the existence of certain bacterial oxidases that would catalyze the transport of electrons between electron donors in the bacteria and a redox dye (tetramethyl-ρ-phenylenediamine dihydrochloride); the dye was reduced to a deep purple color. A piece of filter paper was saturated in a petri dish with oxidase reagent (freshly prepared); and a small portion of the bacterial colonies was spread on the filter paper by a wooden stick. The turning of the colour of the smear from rose to purple within 10 sec indicated a positive result (Forbes *et al*., 2007).

2.2.4.3: Coagulase test

This test was used to detect the ability of tested bacteria to produce the Coagulase which is an enzyme-like protein that clots oxalated or citrated plasma. The test was performed as the following: Citrated human plasma were diluted 1:5 then mixed with an equal volume of an overnight bacterial broth culture, incubated at 37ºC. If clots form in 1-4 hours, the test is positive. A tube of plasma mixed with sterile broth was included as control (Brooks *et al*., 2007).

2.2.4.4: Indole test

This test was performed by inoculating peptone water medium with bacterial growth by the loop, and it was incubated for 24-48 hr at 37 ºC. Indole test was done by adding 6-8 drops of Kovac's reagent (p-dimethyl amino benzaldehyde in amyl alcohol). The positive reaction was characterized by the formation of red color ring at the top of the broth (MacFaddin *et al*., 2000).

2.2.4.5: Methyl –red test

The tubes of the MR-VP broth were inoculated with selective bacterial colonies and were incubated at 37 ºC for 24-48 hr. Five drops of methyl red reagent were then added to it. The appearance and observation of red color means a positive result and a complete hydrolysis of glucose (MacFaddin, 2000).

2.2.4.6: Vogues –proskauer test

The tubes of the MR-VP broth were inoculated with selected bacterial colonies and were incubated at 37 ^{o}C for 24 hr. Then, the result was read by adding 0.6 ml of alpha nephthol (reagent A) and 0.2 ml of 40 % KOH solutions (reagent B). The appearance of red colour after 15 min means a positive result due to the partial hydrolysis of glucose, which produces acetone or acetyl - methyl - carbinol (MacFaddin, 2000).

2.2.4.7: Citrate utilization test

After sterilization of Simmon's citrate medium by autoclaving, the bacterial colonies were inoculated and incubated for 24 hr at 37 ^{o}C, the change of the color of medium from green to blue with streaks of growth indicated positive results, while unchanged original green colour with no growth indicated negative results (Winn *et al*., 2006).

2.2.4.8: Vitec 2 system

The Vitec 2 system was used to confirm the biochemical test and antibiotics assay had been performed according to the manufacturer's instructions. This system consists of personal computer, reader incubator that prepared up of many inner constituents including: card filler mechanism, card cassette, cassette loading processing mechanism, bar code reader, card sealer, cassette carousel and incubator. In addition to transmittance optics, waste processing, instruments control electronics and firm ware. The system was equipped with an extended identification data base for all routine identification tests that provide an improved efficiency in microbial diagnosis

which reduce the need to perform any additional tests, so that will increase safety for both the test and the users.

All the following steps are prepared according to the manufacturer's instructions. Three ml of normal saline are placed in plane test tube and inoculated with a loop full of isolated colony. The colony must be aged 24 hr., the test tube inserted into a dens check machine for standardization of colony to McFarland's standard solution (1.5×10^{8}cell/ml). The standardized inoculums are placed into the cassette and a sample identification number entered into the computer software via barcode. Thus the VITEK 2 card connected to the sample ID number. Then, the cassette is placed in the filler module, when the cards are filled, transferred the cassette to the reader incubator module All following steps handled by the instrument, the instrument controls the incubation temperature, the optical reading of the cards and continually monitors and transfers test data to the computer for analysis (Pincus and David, 2006).

1. Standardization

After primary isolation, handling is minimized in a simple inoculum preparation, standardization and dilution step. The standardized inoculum is placed into the cassette and a sample identification number is entered into the computer software via barcode (Pincus and David, 2006).

2-Traceability

The VITEK 2 card type is then read from the barcode placed on the card during manufacturing and the card is thus connected to the sample ID. Manufacturer barcodes link the card to patient information in this one easy barcode reading step (Pincus and David, 2006).

3-Load and Go

Place the cassette in the filler module. When the cards are filled, transfer the cassette to the reader/incubator module. All subsequent steps are handled by the instrument (Pincus and David, 2006).

2.2.5: Genetic Study

2.2.5.1: Genomic DNA extraction

Favor PrepTM Genomic DNA Mini Kit was used to extract genomic DNA from isolates following the manufacturer's protocol. Inoculum of each isolates was prepared at density up to 109. Bacterial pellets were harvested via centrifugation at 14000 rpm for 1 min. The harvested cells were resuspended thoroughly in 200 µl of FATG buffer (for gram negative isolates) or resuspended in 200 µl of lysozyme (for gram positive isolates) and then, incubated for 10 min at room temperature. buffer FABG (200 µl) was added respectively to the bacterial cells. The resulting homogeneous cells suspension was incubated for 10 minutes at 70ºC and vortexed for 10 sec every 3 min. until the sample lysate is clear. DNA was extracted from the homogeneous suspension by the addition of 96~100% of ethanol and then transferred to the FABG column assembled inside a 2 ml collection tube, and centrifuged for 5 min. at 14000 rpm. The flow through was discarded. The FABG column was placed in a new 2 ml collection tube. W1 Buffer (400 µl) and Wash Buffer (600 µl) were

then added respectively to the FABG column assembled inside a new 2 ml collection tube, and centrifuged for 30 sec at 14000 rpm to wash column membrane. The flow through was discarded. The FABG column was dried via further centrifugation at 14000 rpm for 3 min. over a new 2 ml column collection tube to remove any residual ethanol solution. The dry

FABG column was then transferred to a new 1.5 ml micro centrifuge tube, and 100µl of preheated elution buffer or TE was added directly to the FABG column membrane for 3~5 min, followed by centrifugation for 1 min at 14000 rpm to elute DNA. The purity of eluted genomic DNA was measured by nanodrob, then stored at 20ºC until use.

3.2.5.2: Estimation of DNA concertation

The extracted genomic DNA is checked by using Nano drop spectrophotometer (scan drop, analytical jena, Germany) measure DNA concertation (ng/ml) and checks the DNA purity by reading the absorbance at (260/280 nm).

3.2.5.3: Primer pairs preparation

primer pairs used in this study were dissolved using TE Buffer, (pH 8.0) composed of 10mM Tris-HCl containing 1mM EDTA-Na2. Firstly, the primer stock tube prepared and then the working solution would prepare from primer stock tube. According to the instruction provided by primer manufacturer (Bioneer / Korea) the TE buffer were added to get 100 picomole/microliter concentration of primer stock solution. The working solution prepared from stock by dilution with TE buffer to get 10 picomole/microlite (Green and Sambrook, 2012).

2.2.5.4: Reaction mixture

Amplification of DNA was carried out in a final volume of 50 µl reaction mixture as mentioned in table (2-8).

Table 2-8: Contents of the Reaction Mixture

NO.	Contents of reaction mixture	Volume
1	Green master mix	25 µl
2	Upstream primer (10pmol/µl)	3µl
3	Downstream primer (10pmol/µl)	3 µl
4	DNA template	5 µl
5	Nuclease free water	14 µl
Total volume		50 µl

2.2.5.5: Polymerase chain reaction (PCR)

Conventional PCR were used to amplify the target DNA using specific primer pairs table (2-6) It include three consecutive steps that repeated for specific number of cycles to get PCR product (amplicon) which could be finally visualized after agarose gel electrophoresis. The thermal cycling conditions mentioned in the table (2-6).

2.2.5.6: Agarose Gel Electrophoresis

Agarose gel electrophoresis is the most effective way of separating DNA fragments. The concentration of agarose in a gel depends on the sizes of the DNA fragments need to be separated, ranging between 0.5%-2% (Lee et al., 2012). A 0.7% gel was used to obtain good separation of genomic DNA (5-10 kb) after extraction while 1.5%-2% was used to gain good

resolution for small fragments of PCR product (0.2-1 kb). However, the specific weight of agarose was added to 100ml of 1×TBE buffer and then melted in microwave until the solution becomes clear. Once the agarose was cooled to 50-55°C, 5µl of simply safe dye (10 mg/ml) was added to 100 ml of melting agarose gel to get final concentration 0.5µg/ml (Sambrook and Russel, 2006). The agarose was poured in the gel tray with sealed ends, comb placed properly, and then left to dry. The samples were loaded in a separate well of the gel, with marker in one well. Electrodes were connected correctly and the run was applied according to the gelpercentage and size of gel, (The time of agarose gel electrophoresis is 45 minute for genomic DNA and 1 hour and 30 minute for PCR product).

1. Place the gel-casting tray in plastic tray, check that the teeth of the comb are approximately 0.5mm above the gel bottom.
2. Prepare 500ml of TBE (1X) by adding 50ml of TBE (10X) stock solution to a final volume of 500ml of deionized water.
3. Place 100ml of the buffer into a 500ml flask and add 0.8g of agarose. Melt the agarose by heating (microwave) the solution on hot plate for approximately 10 min. Carefully swirl the agarose solution to ensure that the agarose is dissolved, that is no agarose particles are visible.
4. Cool the agarose solution to approximately 50°C and added 2-3 µl of Simple safe stock solution. Slowly pour the agarose into the gelcasting tray. Remove any air bubbles by yellow tip.
5. Position the comb approximately 1.5cm from the edge of the gel. Let the agarose solidify for approximately 20–30 minutes. After the agarose has solidified remove the comb with a gentle back and forth motion, taking care not to tear the gel.

6. Remove the gel-casting tray and place the tray on the central supporting platform of the gel box.
7. Add electrophoresis buffer to the buffer chamber until it reaches a level of 0.5–1cm above the surface of the gel.
8. Load the samples into the wells using a yellow tip. Place the tip under the surface of the electrophoresis buffer just above the well. Expel the sample slowly, allowing it to sink to the bottom of the well. the sample into a neighboring well. Note: samples must be loaded in sequential sample wells. When loading fewer samples than the number of wells it is preferable to leave the wells nearest the edge of the gel empty.
9. First load 5µl of ladder molecular weight marker to each side of the gel (flanking the sample line) and 20 µl of DNA specimen in the other well.
10.Place the lid on the gel box and connect the electrodes. DNA will travel towards the positive (red) electrode positioned away from the well. Turn on the power supply.
11.Continue electrophoresis until the tracking dye moves at least 10 cm of the gel length.

2.2.5.7: Sequencing of PCR product

forty microliters of PCR product for *Pseudomonas* isolate were send to Macrogene/ Korea for Sanger sequencing. After trimming of each sequence, the result of the trimmed sequence was blasted in NCBI to check the similarities and differences with data base. Mega5 software were used to check the similarities and differences.

2.2.6: Antimicrobial susceptibility test

2.2.6.1: Antibacterial susceptibility test

In this test, the antibiotics names and its standard inhibition diameters were used as it recommended by (CLSI, 2018).

1. The inoculums used in this test were prepared by adding (2-5) isolated colonies grown on nutrient agar plate to 5 ml of sterile normal saline and compared with (1.5X 10^8 cell/ml) McFarland standard tube.
2. A sterile swab was used to acquire inoculums from the bacterial suspension. These inoculums were streaked on a Mueller-Hinton agar (MHA) plate and left to dry.
3. The antibiotics disks were placed on the surface of the medium at consistently spaced intervals with flamed forceps or a disc applicator and incubated for 24 hr. at 37 ^oC.
4. Inhibition zones were measured by using a ruler or a caliper and compared with the zones of inhibition determined by the Clinical Laboratory Standards Institute to conclude the resistance or sensitivity of the organism to each antibiotic.

2.2.6.2: Antifungal susceptibility test

Cycloheximide ($C_{15}H_{23}NO_4$) 50 mg powder, diluted in 1 mL of ethanol, then discs of filter paper impregnated with cycloheximide were applied by disk diffusion method on the surface of the medium (SDA) at evenly spaced intervals with flamed forceps and incubated for 7 days at (25-30 °C) for fungi, individually inoculated with 100 µl of spore suspension (0.1 McF) and then areas of inhibition were measured using a ruler and compared to areas of inhibition defined by (Abu-Mejdad et al., 2014).

2.2.7: Immunological method

2.2.7.1: IL 10 ELISA Kit

2.2.7.1.1: Principle assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human IL-10 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-10 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human IL-10 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-10 bound in the initial step. The color development is stopped and the intensity of the color is measured.

2.2.7.1.2: Reagent preparation

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Calibrator Diluent RD5C (diluted 1:5) - Add 20 mL of Calibrator Diluent RD5C Concentrate to 80 mL of deionized or distilled water to yield 100 mL of Calibrator Diluent RD5C (diluted 1:5).

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Human IL-10 Standard - Refer to the vial label for reconstitution volume. Reconstitute the Human IL-10 Standard with deionized or distilled water. This reconstitution produces a stock solution of 5000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 900 µL of Calibrator Diluent RD5C (diluted 1:5) (for cell culture supernate samples) or Calibrator Diluent RD6P (for serum/plasma samples) into the 500 pg/mL tube. Pipette 500 µL of the appropriate calibrator diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 500 pg/mL standard serves as the high standard. The appropriate calibrator diluent serves as the zero standard (0 pg/mL).

2.2.7.1.3: Assay procedure

The reagents and samples were brought to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

1. Prepared all reagents, working standards, and samples were as directed in the previous sections.

2. Removed excess microplate strips were removed from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal. 3. For Serum/Plasma Samples Only: Add 50 µL of Assay Diluent RD1W to each well.

4. Added 200 µL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Added 200 µL of Human IL-10 Conjugate to each well. Cover with a new adhesive strip. For Cell Culture Supernate Samples: Incubate for 1 hour at room temperature. For Serum/Plasma Samples: Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 200 µL of Substrate Solution to each well. Protect from light. For Cell Culture Supernate Samples: Incubate for 20 minutes at room temperature. For Serum/Plasma Samples: Incubate for 30 minutes at room temperature.

9. Added 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction

will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate

2.2.7.2: IFN-γ ELISA Kit

2.2.7.2.1: Principle assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human IFN-γ has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IFN-γ present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human IFN-γ is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IFN-γ bound in the initial step. The color development is stopped and the intensity of the color is measured

2.2.7.2.2: Reagent preparation

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5P (diluted 1:5) - Add 5 mL of Calibrator Diluent RD5P to 20 mL of deionized or distilled water to prepare 25 mL of Calibrator Diluent RD5P (diluted 1:5).

Human IFN-γ Standard - Refer to the vial label for reconstitution volume. Reconstitute the Human IFN-γ Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Pipette 900 µL of Calibrator Diluent RD5P (diluted 1:5) into the 1000 pg/mL tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. Calibrator Diluent RD5P (diluted 1:5) serves as the zero standard (0 pg/mL).

2.2.7.2.3: Assay procedure

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

1. Prepared all reagents and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1-63 to each well.
4. Add 100 µL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. Ensure

reagent addition is uninterrupted and completed within 15 minutes. A plate layout is provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or auto washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Added 200 µL of Human IFN-γ Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

7. Repeated the aspiration/wash as in step 5.

8. Added 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. Protect from light.

9. Added 50 µL of Stop Solution to each well. The color in the well should change from blue to yellow. If the color in the well is green or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

2.2.7.3: CTLA-4 ELISA kit

2.2.7.3.1: Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human CD152 antibody CD152 present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human CD152 Antibody is added and binds to CD152 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated CD152 antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human CD152. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

2.2.7.3.2: Reagent Preparation

All reagents should be brought to room temperature before use. **Standard** Reconstitute the 120µl of the standard (80ng/ml) with 120µl of standard diluent to generate a 40ng/ml standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (40ng/ml) 1:2 with standard diluent to produce20ng/ml, 10ng/ml, 5ng/ml and2.5ng/ml solutions. Standard diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C and used within one month.

Wash Buffer Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

2.2.7.3.3: Assay Procedure

1. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.

2. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C. 3. Add 50µl standard to standard well. Note: Don't add antibody to standard well because the standard solution contains biotinylated antibody.

4. Added 40µl sample to sample wells and then add 10µl anti-CTLA-4 antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.

5. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate or decant each well and wash 5 times with wash buffer. Blot the plate onto paper towels or other absorbent material.

6. Added 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.

7. Added 50µl Stop Solution to each well, the blue color will change into yellow immediately.

8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

2.2.7.4: Calculation of Results

The ELISA results were calculated depend on the optical density reading for each standard and samples optical density. Then the standard curve was plotted by the mean OD value for each standard on the Y-axis against the concentration on the X-axis and draw a best fit curve through the points on the graph.

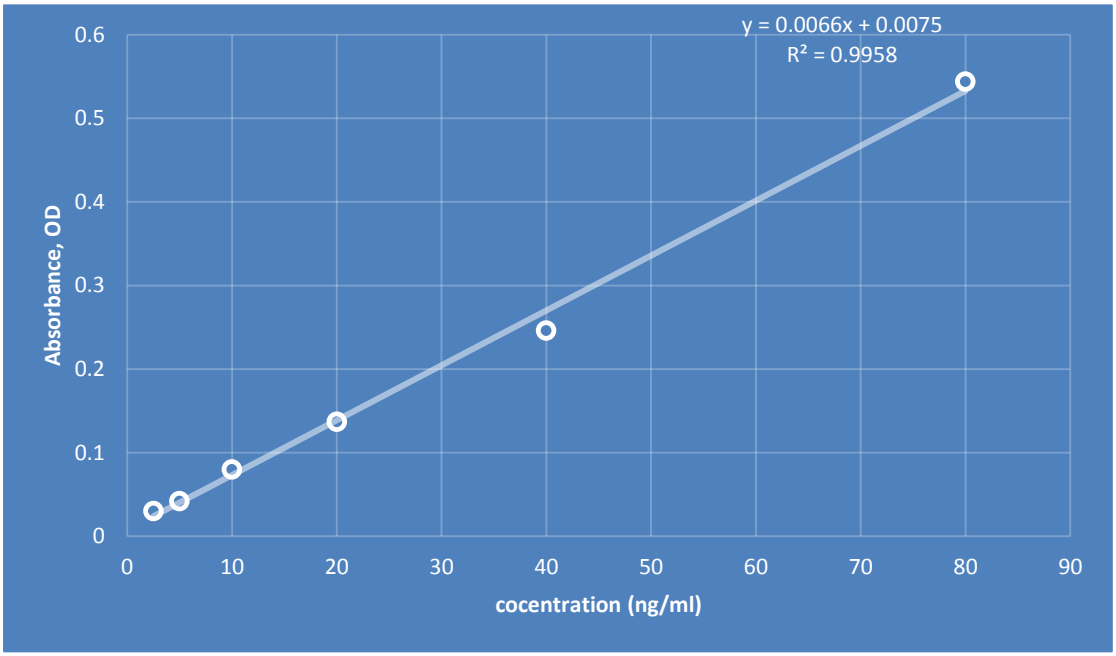

Figure (2-2): Human Cytotoxic T Lymphocyte Associated Antigen 4 (CTLA-4) Standard Curve that showed the Concentration (ng/ml) and

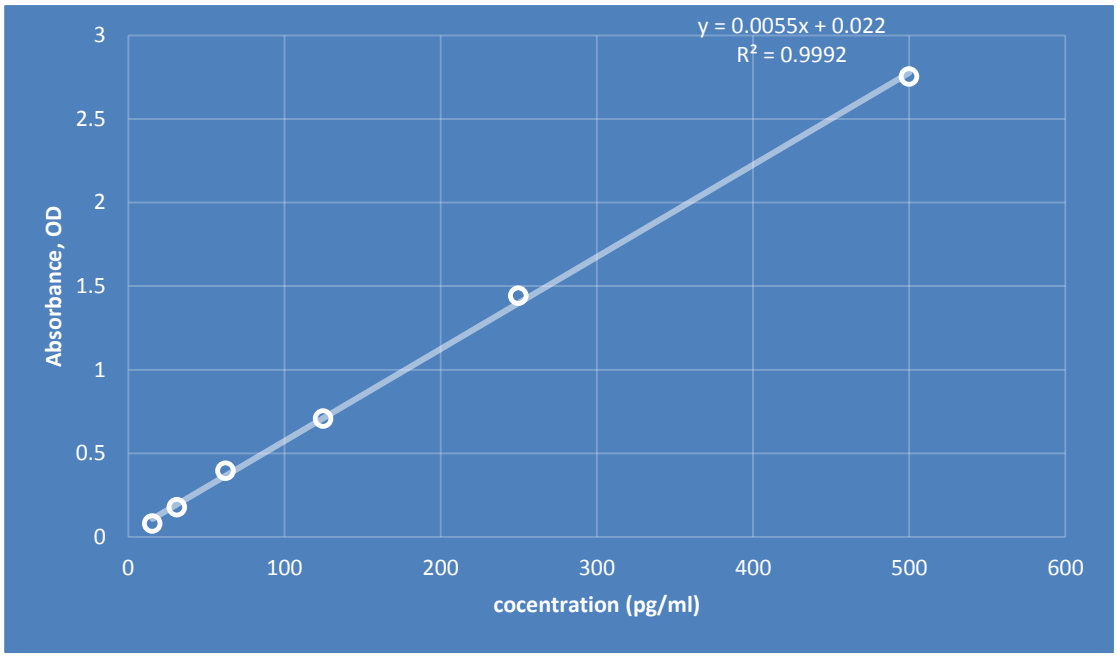

Figure (2-3: The human Interleukin 10 (IL-10) Standard Curve that showed the Concentration (ng/ml) and optical density OD: 450nm

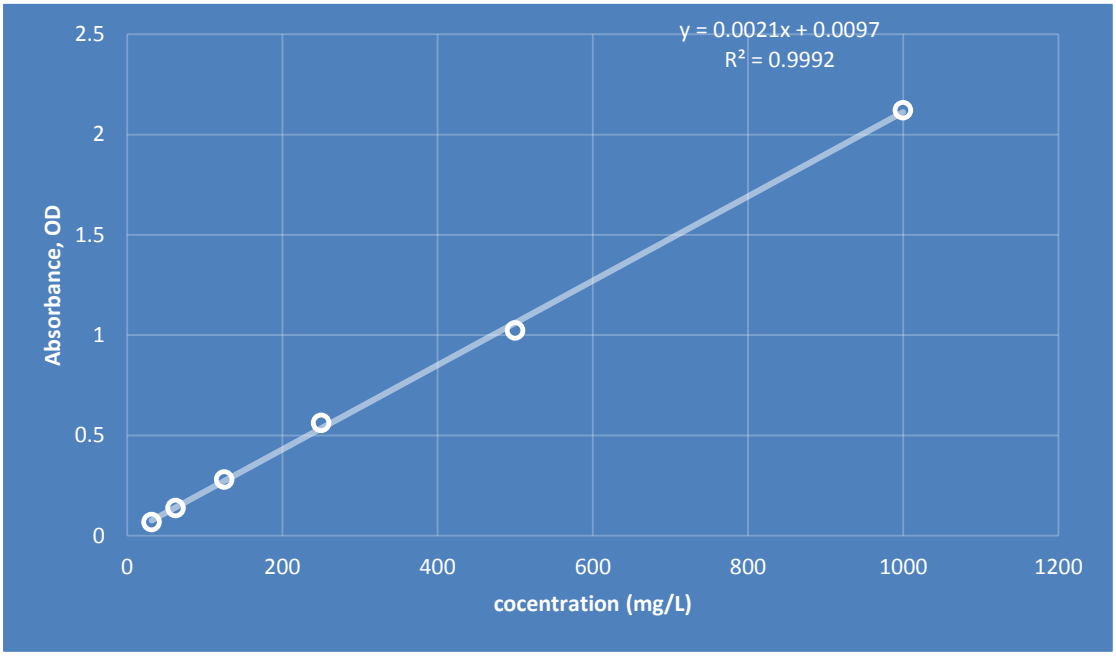

Figure (2-4): The human interferon gamma (IFN-γ) Standard Curve that showed the Concentration (ng/ml) and optical density

2.2.8: Biosafety and Hazard Material Disposing

Biosafety aspects followed during the work include disposing of all swabs, Petri dishes and all contaminated supplies by autoclaving and then incineration. All benches cleaned with alcohol before and after the work.

2.2.9: Statistical Analysis

Data was analyzed using SPSS (version 23, SPSS Inc. Chicago, Illinois, USA). Descriptive statistics (mean, standard Error), and differences were compared by One-way ANOVA at $p \leq 0.05$ by using Duncan's test.

CHAPTER THREE

RESULTS and DISCUSSION

3: Results and Discussion

3.1: Study Population of Patients with Otitis Media and Control According to Gender and Age Groups

A total of (90) blood samples and ear discharge by a sterile transport medium swab were collected from patients with otitis media aged between(1 - 80 years) they were suffering from otitis media in ENT Department of AL-Hilla Teaching Hospital and Imam Sadiq Hospital in Babylon city after clinically diagnosed by consultant physician during the period between October 2021 to February 2022. Thirty (30) blood samples as control which are apparently healthy age marched groups for each 15 male and 15 female.

A total of (90) male and female were distributed into eight groups according to the age, 1 – 9 years (15) patients, 10 – 19 years (12) patients, 20 – 29 years (14) patients, 30 – 39 years (11) patients, 40 –49 years (10) patients, 50 –59 years (9) patients, 60 – 69 years (9) patients and 70 – 80 years (10) patients as shown in Table (3-1) and Figure (3-1).

The results showed that the highest rate of infection was in the first age group (1 – 9 years) in the rate of (16.6%) , the third age group (20 – 29 years) in the rate of (15.5%) , followed by The second age group (10 – 19 years) in the rate of (13.3%) , the fourth age group (30 – 39 years) in the rate of (12.2%) ,The fifth age groups (40 –49 years) and the eight age groups (70 – 80 years) in the rate of (11.1%) , the sixth age group (50 –59 years) and the seventh age groups (60 – 69 years) in the percentageof infection was (6.6%).

The highest rate of infection in the first age group is due to incomplete immune system especially in children and weak physical structure (DeAntonio *et al.*, 2016).

Actually, this observation is in agreement with previous authors who stated that the onset of otitis media is more common in children (Revai et al., 2007; Macintyre et al., 2010; Kaur et al., 2017).

Table (3-1): Gender and Age Groups Distribution of Otitis Media

No.	Age years	Male	Female	Total	percentage
1	**1-9**	**10**	**5**	**15**	**16.6%**
2	**10-19**	**4**	**8**	**12**	**13.3%**
3	**20-29**	**8**	**6**	**14**	**15.5%**
4	**30-39**	**5**	**6**	**11**	**12.2%**
5	**40-49**	**5**	**5**	**10**	**11.1%**
6	**50-59**	**4**	**5**	**9**	**6.6%**
7	**60-69**	**5**	**4**	**9**	**6.6%**
8	**70-80**	**7**	**3**	**10**	**11.1%**
	Total	**48**	**42**	**90**	**100%**
	Control	**15**	**15**	**30**	**100%**

According to previous literatures, the peak incidence of otitis media occures between 6 and 12 months of life and becomes less after the age of five and it is less common in adults than in children. Around 4 fifths of all children will have a case of otitis media throughout their lifetime, and between 80 to 90% of all children will have otitis media with effusion before attending school. Otitis media (Usonis et al., 2016; Schilder et al., 2016).

The most acceptable explanation for the higher incidence of otitis media in children is the high prevalence of acute upper respiratory tract viral infections in children in comparison with adults. In one previous Iraqi study (Alansary et al., 2016), history of upper respiratory tract infection was reported in most of children (88.3 %) who were suffering from otitis media with effusion.

The viral upper respiratory tract infections will disrupt the ciliary activity of Eustachian tube leading to the loss of the mechanism that normally prevents bacteria from reaching the middle ear. Therefore, bacteria can reach the middle and can cause colonization and establishment of infection secondary to upper respiratory tract infection (Revai et al., 2007). The other possible explanation for the higher incidence rate of otitis media in children in comparison with adult is the less well-developed immune system in children in comparison with adults (Revai et al., 2007; Sharma et al., 2013; Mittal et al., 2014).

As for the lowest rate of infection was in the seventh age group and the sixth age group (6.6%), due to the lack of auditors from this category to the advisory clinic because of age, and the results of this study are compatible with （Kumar *et al.* ,2013).

The male percentage was different than female in otitis media as in the patients were distributed as 48 male percentage (53.3%) and 42 female percentage (46.7%). The current study, comparison of between male patients with otitis media and male control group revealed that the level was higher in male patients' group in a highly significant manner ($p < 0.0001$), while comparison of between female patients with otitis media and female control group revealed that the level was higher in male patients' group in a Less significant ($p < 0.001$) as illustrated

in Table (3-2).

This is due to the fact that males are more susceptible to environmental conditions in their lives such as swimming pools or in other rivers and ponds than females, and this result is consistent with other results (Kumar *et al.* ,2013; Kadhim *et al.* ,2018).

Table (3-2): Comparison of frequency distribution of individuals according to gender between patients with otitis media and control group.

Characteristics Gender	Patients *n* = 90	Control *n* = 30	p-value
	No.(%)		
Male	48 (53.3%)	15 (50.0 %)	≤0.0001**
Female	42 (46.7%)	15 (50.0 %)	0.001**
Male: female ratio	1.2:1	1:1	

n: number of cases,

. * significant difference at P≤0.05

, ** significant difference at P≤0.01

3.2. Disease Characteristics of Patients with Otitis Media

3.2.1. The Frequency Distribution of Patients with Otitis Media According to Mode of Clinical Presentation

The frequency distribution of patients with otitis media according to mode of Clinical presentation is shown in table (3-3) most of patients in the current study had acute otitis media (AOM) accounting for 20 % followed by chronic suppurative otitis media (CSOM) accounting for 73.3 % and lastly by otitis media with effusion (OME) accounting for 6.6 % only, when comparison of between acute otitis media, chronic suppurative otitis media and otitis media with effusion with otitis media are highly significant difference at ($p \leq 0.0001$) can be observed in table (3-3).

Table (3-3): The frequency distribution of patients with otitis media according to mode of clinical presentation.

Type of Inflammation	Number of cases	%
AOM	18	20
CSOM	66	73.3
OME	6	6.6
p-value	**≤0.0001****	

AOM: acute otitis media; **OME**: otitis media with effusion; **CSOM**: chronic suppurative otitis media

. * significant difference at P≤0.05,

** significant difference at P≤0.01

The inflammation of otitis media is categorized according to severity of clinical presentation, duration of disease and associated clinical manifestations into chronic suppurative otitis media (CSOM), acute otitis media (AOM), chronic otitis media (COM) and Otitis media with effusion (OME). chronic suppurative otitis media is characterized The incidence of CSOM varies from 1.7 to 9.4 cases per 1,000 people and OM-related tympanic membrane rupture can lead to temporary or permanent hearing loss, which can affect social interactions and developmental outcomes in childhood in rare cases, the infection can lead to serious complications such as meningitis mastoiditis, and intracranial and extracranial abscesses. (Perdrizet et al., 2022).

The prevalence ratio of active CSOM in this study was 73.3%. This ratio was very high because most of the patients with hearing impairment who visit in ENT Department These results are similar to what the following research said, hearing impairment often accompanies this disease. People are at increased risk

of developing CSOM when they have poor Eustachian tube function, a history of multiple episodes of acute otitis media, live in crowded conditions, and attend pediatric day care facilities, Worldwide approximately 11% of the human population is affected by AOM every year, or 709 million cases About 4.4% of the population develop CSOM(Peterson et al., 2012, Vos et al., 2015) , According to the World Health Organization, CSOM is a primary cause of hearing loss in children (Vos et al., 2015). Adults with recurrent episodes of CSOM have a higher risk of developing permanent conductive and sensorineural hearing loss (Yunusa and Mansoor, 2022).

The incidence of CSOM across the world varies dramatically where high income countries have a relatively low prevalence while in low income countries the prevalence may be up to three times as great.(Peterson et al., 2012) Each year 21,000 people worldwide die due to complications of CSOM(Acuin et al, 2004).[

While I found a study in the city of Hilla saying that the highest percentage of acute otitis media (AOM) 76.7% , chronic suppurative otitis media (CSOM) 23.3% and otitis media with effusion (OME) 8.3 % (Abed et al., 2021), which explains this current study is the transformation of patients' condition from ,acute otitis media (AOM) to chronic otitis media (COM).

Where it was found in this current study a clear decrease in acute otitis media 6.6 % and it is consistent with (Eythorsson et al., 2018)visits for AOM decreased from 47.5 to 33.9 visits per 1000 person-years, incidence rate ratio.

Number of patients with AOM decreased during 2011–2013 and leveled off during 2014–2018(Kono et al., 2021).

Due to education efforts and promotion of the proper use of antimicrobials through means such as the Clinical practice guidelines for the diagnosis and management of acute otitis media in children (2006) and the Manual of Antimicrobial Stewardship (2016), a change in the use of antimicrobials occurred, leading to a trend to more proper use of these agents(Kono et al., 2021).

There generally great difficulty in the estimation of the true incidence or prevalence rate of otitis media with effusion because it is usually asymptomatic however previous estimates according to large cohort studies have shown that OME accounts for 20 % and that children are mostly affected (Schilder et al., 2015). The low rate of OME globally is therefore in line with the low rate of OME seen in the present study and the reason is the same as most patients are asymptomatic and do not seek medical advice.

3.3: Microbes Identification Isolated from Patients with Otitis Media

3.3.1: Bacterial Identification

3.3.1.1: Phenotypic Identification

To study the morphological features of microbial isolates several methods were used, a group of microorganisms have been identified including bacteria. The colony form was adopted as an initial diagnosis, staining smear with Gram stain bacterial was made to study microscopic characteristics observed under oil immersion lens (100X). The bacterial isolate was appearing as Gram positive and Gram negative (cocci, coccobacilli and rod), so the biochemical test (Appendix, Table 5, and 6, Figure 2 and 3) depending to the Winn *et al.* (2006), and Vitek 2 compact system was confirmed the final diagnosis (Appendix, Table 6, and 7).

Ear swabs were subjected for culturing on different types of culture media, to diagnosis the cause agents , the results of cultured medial revealed that (64) samples gave positive microbes culture, whereas (26) samples showed no bacterial growth even after 48 hours, which may due to consumption of antibiotics by the patients or the presence another types of causative agents, that might need special technique for their detection such as virus, parasite, *Chlamydia* and *Mycoplasma* (Heikkinen and Chonmaitree, 2003) .

A total of (90) samples, where (26) samples no growth, while (64) samples were obtained from (64) culture positive isolates consist of bacterial isolates were obtained from the (90) samples collected, (15) fungi from mixed isolates were obtained from (15) samples collected (fungi with bacteria). From the results, it was shown that Gram positive bacteria constitutes (45.5%)(41/90) from the total isolates and were considered the predominanta etiological agent of OM compared to Gram negative bacteria which constitute (25.5%) (23/90) while fungi constitutes (16.6%) (15/90) As shown in Figure (3-2) and (3-3).

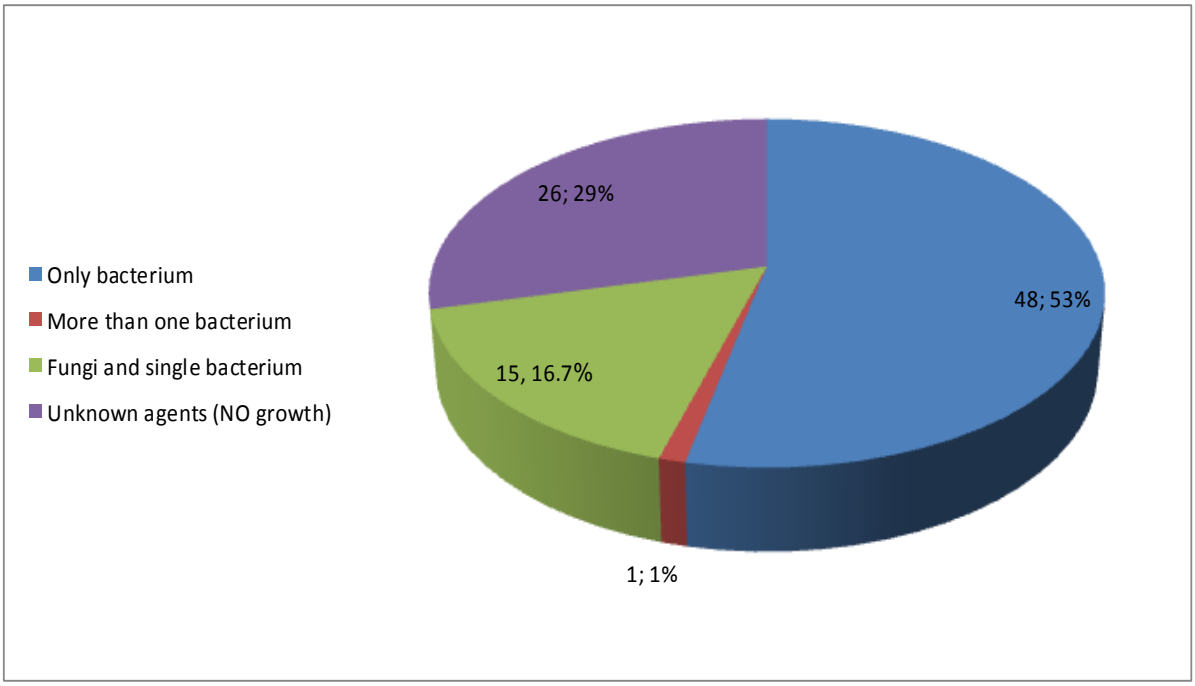

Figure (3-2): Number and percentage of type causative agents isolate from patients with Otitis Media.

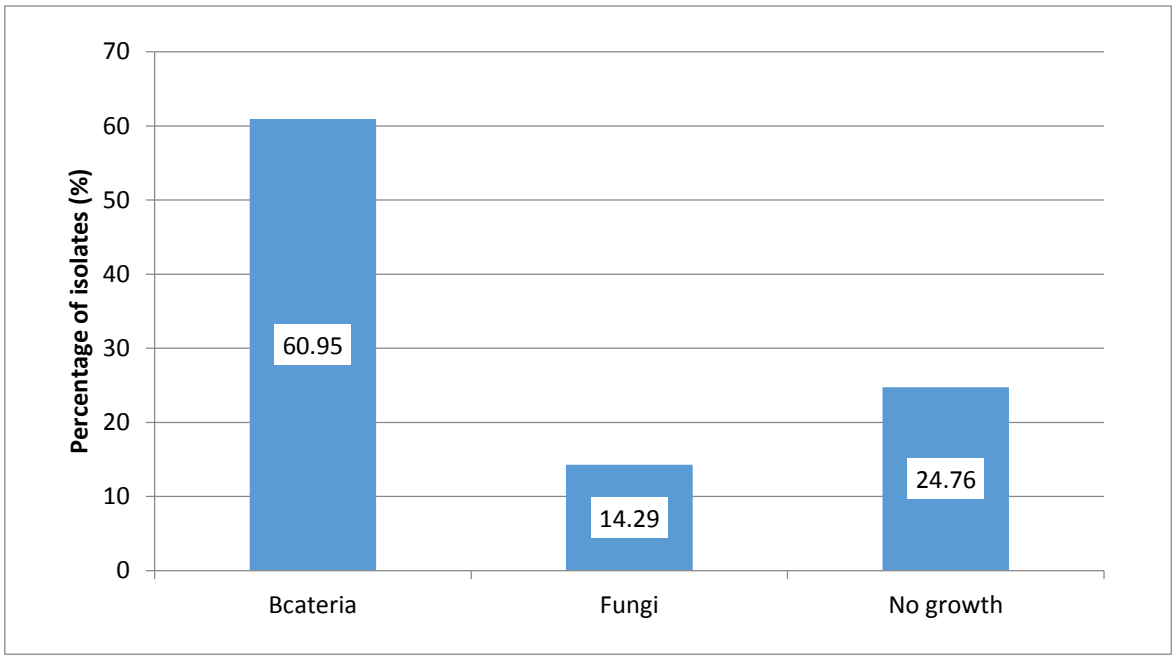

Figure (3-3): Number and Percentage of Microorganisms Isolates fromOtitis Media Patients.

In the present study out of 90 cases, had pure culture in which bacterium and fungi (48(53.3%) and 15 (16.7%) cases) respectively, 26 (28.8%) cases did not show any growth, 1 (1.1%) cases more than one bacterium, 15 (16.7%) cases had mixed culture show in Table (3-4).

The results were shown the pure culture in which bacterium higher than others causative agents (Bhan *et al.*, 2016).

The bacterial infection higher than others which was (53.3%) while mixed (fungi with bacterial) because infection was (16.7%), fungi and unknown infection was (28.8%) in patients which shown in Table (3-5), differences in types of isolated infection depend on different types of environmental factors, geographical regions and health of people. This result might be shown that mixed causative agents are belonging to two types bacterium to bacterium as primary infection bacterium and caused secondary causative agent with other fungi causes, also due to secondary infection as complication of primary bacterium infection. This groups of patients are critical to diagnosis which one of agents are first causative from other (Jreemich, 2017).

Table (3-4): Main Causative Groups of Microorganisms according of age.

No. of patients / Causative agent	Total	Age groups				%
		1-20	21-40	41-60	61-80	
Bacterial	48	19	14	12	3	53.3%
Mixed bacteria	1	0	1	0	0	1.1%
Mixed bacteria and Fungi	15	6	1	0	8	16.7%
Unknown	26	10	6	8	2	28.8%
Total	90	35	22	20	19	100%

This result might be referring to that, the bacterial causes are more current than other and concluded that mixed group have fungi associated otitis media as well as the unknown group is to related to other causative agents not included in present study (Jain *et al.*, 2010). Similar to this current study.

The main bacterial types isolated in this study were shown in table (3-5). *S. aureus, Ps. aeruginosa* and *S.epidermidis* were the most common bacterial species isolated from ear infection (37.7% , 7.7% and 6.6%) respectively were bacterial isolates. From the total bacterial isolates, 57% were gram-positive bacteria, while gram-negative bacteria were 43%; with predominant of Pseudomonas aeruginosa, (22%) (Agha and Al-Delaimi, 2021).

S. aureus was the first main type of bacteria isolated from otitis media (37.7%), study in the city of Dohuk in 2018 is Staphylococcus aureus (19%)(Agha and Al-Delaimi, 2021). this frequency may be due to firstly, it may enter the middle

ear from external canal as a normal flora and by reflux OM when the tympanic membrane was not intact, secondly, *S. aureus* also contain teichoic acid and lipoteichoic acid, capsular material which facilitates the adherence of these bacteria to epithelium this agree with the result mentioned by (Emikpe and Oyero, 2007).

Staphylococcus aureus (S. aureus) is known to be a major cause of skin and soft tissue infections, pneumonia, bloodstream infections, and infective endocarditis via nosocomial transmission in hospitals, in otorhinolaryngology, S. aureus infection has become a great concern due to its ease of its propagation via medical appliances used to evaluate the nasal and oral cavities, in previous studies, the prevalence of S. aureus in ear discharge was reported to range from 9.9% to 54.1%*(Hwang et al., 2015).*

S. epidermidis was previously regarded as non-pathogenic as they considered as contaminants from the skin. This agrees with (Saadobi *et al*. 2010), who had emphasized their essential role in chronic OM. These commensals bacteria may have a role as opportunistic pathogens in thepresence of weakened local tissue defense when immune suppressive agents were used, and also the antibiotics had been associated with the emergence of opportunistic infection by microorganisms not previously regarded as pathogenic to main like *S. epidermidis* and Diphthroid bacilli.

However, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the most common aerobic microbial isolates in patients with CSOM, followed by *Proteus vulgaris* and *Klebsiella pneumoniae* (Aduda *et al.,* 2013). A number of studies from different countries including Iraq (the city of Ramad) ,iIndia, Nepal, Singapore and Nigeria have reported that *P. aeruginosa* is the most common pathogen that causes CSOM, followed by *S. aureus* ((Al-Ani et al., 2021).

Pseudomonas aeruginosa was the main second type of bacteria isolated from otitis media (7.7%). P. aeruginosa is the most common bacteria isolated from chronic supportive otitis media This finding is in tandem with the observations

by other studies (20). Our study was disagreement with other studies done by (21,22), that reported staphylococcus aureus was predominant causative agent in CSOM followed by P. aeruginosa.(Ghali et al., 2021).

Pseudomonas aeruginosa and *Staphylococcus aureus* were pathogens most frequently identified from the ear in the course of chronic otitis media. *Pseudomonas aeruginosa* was concerned with major pathology of the middle ear (radical surgery, cholesteatoma or granulomatous tissue, persisting discharge after treatment), whereas *Staphylococcus aureus* was obtained in dry perforations without other pathology in the middle-ear cavity(Kaźmierczak et al., 2022).

Pseudomonas aeruginosa is the most invaders to middle ear when the resistance is lowered and *Ps. aeruginosa is* not necessary to be by the nasopharyngeal stream but may be through fecal contamination of the auditory stream. *Pseudomonas* have many reasons to affect the middle ear firstly, it spread easily to the compromised patients via external canal from healthy carriers or environmental sites, as swimming in rivers and swamps itself one of the important ways to the occurrence of otitismedia with *Ps. aeruginosa* and enteric bacteria, where these water sources are vulnerable to contamination with waste of human and animal, which is an important source of environmental pollution sources (Kadhim et al. , 2018).

Secondly, the high incidence of *Ps. aeruginosa* indicates more general antibiotic resistance than in the case with Gram positive strains (Due to the widespread and indiscriminate use of manyantibiotics in the treatment of cases of middle ear inflamm ation) (Dragojlović *et al.* ,2002). Thirdly, its resistance for phagocytosis by producing a large number of extracellular products such as alkaline protease, elastase and exotoxin A which can cleave Tri-chloroacetic acid（TCA） lead to inhibit the function of immune system of the cells .Fourthly, another way that helps to transfer pathogens to the middle ear is to use incorrect methods for cleaning the external ear (Kadhim *et al.* , 2018).The results were in agreement with (Nia *et al.* , 2011) who had found that *Pseudomonas aeruginosa*

presence in (7.7%) in their study.

Klebsiella pneumoniae and *E. coli* in (4.4%) and (6.6%) respectively as shown in Table (3-5), the presence of these bacteria may be due to presence of capsular polysaccharide which plays an important role in pathogenicity of *Klebsiella pneumoniae* in OM infection, the finding is in inagreement with Klebsiella pneumonia(15.5%) while agreement with E. coli(6.9%) (Poorey and Thakur, 2015) , who found that microorganisms were representing in 15.5% and 6.9% respectively, and the results (5.5%) and (3.4%) respectively as shown with (Wan Draman et al., 2021) .

Other Gram negative bacteria *Acinetobacter ursingii* were isolated from OM in (2.2%) shown in Table (3-5). while (Wan Draman et al., 2021) , mentioned that Acinetobacter represent with (4.5%).

In this study *Proteus mirabilis* constitute (1.1%) as shown in Table (3-7), and was considered the sixth bacteria of Gram negative isolated in this study. This finding was compatible with (Wan Draman et al., 2021), *Proteus mirabilis* represented (3.8%) and incompatible with(Poorey and Thakur, 2015).

Several studies have indicated that the condition of the middle ear infection of the Gram positive bacteria is derived from the nasal pharyngeal cavity is predominant, either the source of Gram negative bacteria to the intestinal is not the nasal pharyngeal cavity, but the contamination of the auditory channel of this bacteria may be the cause of the case of injury (Könönen *et al.* , 2002) .

Table (3-5): Distribution of Microbial Isolates from Patients with OtitisMedia According to The Type.

Type Causes	Microbial	Single Isolates	Mixed More Than Bacteria	Mixed Bacteria + Fungi	Total Isolate	%	NO.
G +ve	*Staphalococcus aureus*	20	0	14	34	37.7	41 45.5%
	Staphalococcus epidermidis	6	0	0	6	6.6	
	Streptococcus pyogenes	1	0	0	1	1.1	
G –ve	*Pseudomonas aeruginosa*	7	0	0	7	7.7	23 25.5%
	Escherichia coli	4	1	1	6	6.6	
	Klebsiella pneumoniae	4	0	0	4	4.4	
	Enterobacter cloacae	3	0	0	3	3.3	
	Acinetobacter ursingii	1	1	0	2	2.2	
	Proteus mirabilis	1	0	0	1	1.1	
Type Causes	Microbial	Single Isolates	Mixed With One Bacteria	Mixed With More Bacteria	Total Isolate	%	NO.
Fungi	*Aspergillus niger*	0	1	0	1	1.1	15 16.6%
	Yeast						
	Candida parapsilosis	0	10	0	10	11.1	
	Malassezia furfur	0	4	0	4	4.4	

Table (3-6): Distribution of Microbial Isolates from Patients with Otitis Media according to The Type Otitis Media

Type Causes	Microbial	AMO	CSOM	OME	Total Isolate	%	NO.
G +ve	*Staphalococcus aureus*	5	27	2	34	37.7	41 45.5%
	Staphalococcus epidermidis	2	3	1	6	6.6	
	Streptococcus pyogenes	1	0	0	1	1.1	
G –ve	*Pseudomonas aeruginosa*	2	5	0	7	7.7	23 25.5%
	Escherichia coli	1	4	1	6	6.6	
	Klebsiella pneumoniae	0	3	1	4	4.4	
	Enterobacter cloacae	1	2	0	3	3.3	
	Acinetobacter ursingii	1	1	0	2	2.2	
	Proteus mirabilis	1	0	0	1	1.1	
Fungi	*Aspergillus niger*	1	0	0	1	1.1	15 16.6%
	Yeast						
	Candida parapsilosis	4	6	0	10	11.1	
	Malassezia furfur	0	3	1	4	4.4	
NO growth	Unknown agents	4	21	1	26	28.8	26 28.8%

3.3.1.2: Molecular Identification Polymerase chain reaction (PCR)

The molecular diagnosis method was used to confirm the phenotypic identification, where the most common samples were used, a sample of gram positive bacteria (Staphalococcus aureus) using a special primer (16S rRNA primer pair) and a sample of gram negative bacteria (Pseudomonas aeruginosa) using the Sequencing of PCR (DNA sequence analysis) which was found to match the results.

Figure (3-4): (1.5 %) Agarose gel electrophoresis at 72 volts for 80minute of PCR to Ps.spp amplicon (791bp), (1) represented Pseudomonas aeruginosa isolates, M DNA marker size (100bp), (2) represented Staphalococcus aureus isolates, Ps.spp amplicon (1498bp).

3.3.1.2.1: Sequencing of PCR product

The forty microliters of PCR product for Pseudomonas isolate were send to Macro gene/ Korea for Sanger sequencing. After trimming of each sequence, the result of the trimmed sequence was blasted in NCBI to check the similarities and differences with data base. Mega5 software were used to check the similarities and differences.

Table (3-7): Alignment of S11 Pseudomonas aeruginosa strain G_8_F-F06 16S ribosomal RNA gene, partial sequence ID: KU314419.1

Score	Expect	Identities	Gaps	Strand
1192 bits(645)	0.0	645/645(100%)	0/645(0%)	Plus/Plus

```
Query  2
GGCCTACCATGCAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGG  61

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16
GGCCTACCATGCAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGG  75

Query  62
TGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAAT  121

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  76
TGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAAT  135

Query  122
ACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGC  181

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  136
ACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGC  195

Query  182
CTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGG  241

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  196
CTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGG  255

Query  242
TCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA  301

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  256
TCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA  315
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Query  302
GCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG  361

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  316
GCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG  375

Query  362
AAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTG  421

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  376
AAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTG  435

Query  422
CTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAA  481

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  436
CTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAA  495

Query  482
TACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAG  541

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  496
TACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAG  555

Query  542
CAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTA  601

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  556
CAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTA  615

Query  602  GAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATG  646
             |||||||||||||||||||||||||||||||||||||||||||
Sbjct  616  GAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATG  660
```

3.3.1.3: Antibiotic susceptibility test of bacterial isolates:

Some antibiotics were used to show the effect on different types of microbes isolated from OM infected patients. Disc diffusion method was used in study the sensitivity of bacterial to antibiotics and Vitek 2 compact system (Appendix, Table 8 and 9). It has been found that there is clear variation in resistance, and most isolates shown resistance to one or more of these antibiotics.

In present study the (*Staphylococcus aureus*) isolate were appeared to sensitive Ceftriaxone and Erythromycin (73.5% and 70.5%) respectively. The results were in agreement with (Wan Draman et al., 2021). who had found that (93%) of *Staphylococcus aureus* were sensitive to Ceftriaxone while (80%) were sensitive to Erythromycin. This finding was in agreement with (Aldhaher et al., 2018)conducted in Al-Kindy Teaching Hospital / Baghdad /Iraq.

It has been found that most of these isolates were high resistant to beta lactam group Amoxicillin + Clavulanic acid (73.6%) and Penicillin (70.7%) as shown in Table (3-8). This result in almost identical with those obtained by (Mukhopadhyay *et al*., 2003), who have pointed these bacteria produce chromosomally encoded beta lactamases that mediated resistance to beta-lactam and Cephalosporin, and also by reducing permeability of these antibiotics inside the cell by alterations in the outer membrane proteins (porins). This result of high resistance to Amoxicillin and Clavulanic acid and Cefotaxime were nearly compatible with that of (Kehinde et al. 2004), who found that S. aureus were resistant to Ampicillin and Cloxacillin (beta-lactam antibiotics) (Al-Saedi ,2000). found that Staphylococcus aureus to Penicillin resistant (13.3%) with (Wan Draman et al., 2021). This finding was inagreement with conducted in(Garg et al., 2022).

Addition of β-lactam inhibitor (such as Clavulanic acid) to the β-lactam antibiotics has been partially successful in alleviating the resistance. Some bacteria have developed mutated Penicillin binding proteins and thus have developed resistance to all of the β-lactam antibiotics (except the newest Cephalosporin antibiotics, Ceftobioprol and Ceftaroline) (Ehmann et al., 2012).

It has been found that most of these isolates were high sensitive Gram negative bacteria (Pseudomonas aeruginosa) is Amikacin (85.7%) and Gentamicin (71.4%) antibiotics also bacteria Klebsiella pneumoniae were high

sensitive Amikacin (75%) and Gentamicin (100%) antibiotics as shown in Table (3-9) , The results were in agreement with (Garg et al., 2022, Sharma et al., 2018), This finding was inagreement with (Wan Draman et al., 2021). Therefore, in this current study, we recommend the use of Amikacin against Pseudomonas aeruginosa.

The resistances of *Pseudomonas aeruginosa* isolates to pencillin may bedue to the synthesis of β-lactamase as well as loss of pencillin binding protein (PBP) by mutation confer resistance to this antibiotics *Pseudomonas aeruginosa*exhibits resistance to many antibiotics possibly due to the intrinsic or acquired inwhich these bacteria are highly inherently resistance and this arises from combi-nation of unusually restricted outer membrane permeability and chromosomallyencoded β-lactamase. This agrees with result mentioned by (Bisiklis *et al*. ,2005).Resistance to multiple antibiotics including Amoxicillin + Clavulanic acid, Cefotaxime, Aztreonam and others have generally increased among a number ofGram negative hospital pathogens especially *K. pneumoniae* , *Enterobacter spp.*, *Pseudomonas aeruginos*a and *Acinetobacter spp*. (Al-Gibouri , 2006).

Escherichia coli isolates were high sensitive to is Amikacin (83.3%) and Ceftriaxone (66.6%) antibiotics, Enterobacter cloacae isolates were high sensitive to is Amikacin (100%) antibiotics The results were in agreement with(Prakash et al., 2013) , This finding was inagreement with(Osazuwa et al., 2011).

Proteus mirabilis is sensitive to Gentamicin , Ceftriaxone and Amikacin The results were in agreement with(Onifade et al., 2018) , This finding was inagreement with (Hassan and Adeyemi, 2007).

The effect of Amoxicillin on *Proteus spp.* was studied as shown in the

results showed that (100%) of isolates were resistant to Amoxicillin + Clavulanic acid. These results of resistant of this antibiotic are nearly compatible with that of (Al-Jumaa *et al.* 2011), who found that (90%) of *Proteus spp.* were resistant to Amoxicillin.

Proteus spp. were resistant to Amoxicillin as a results to their production of β-lactamase enzymes that breakdown the β-lactam ring and under inactive (Chanal *et al*., 2000).

Table (3-8) showed antibiotics sensitivity against Gram positive bacteria isolates from Otitis Media

Antibiotics	*Staphylococcus aureus*		*Streptococcus pyogen*	
	Sensitivity (%)	Resistance (%)	Sensitivity (%)	Resistance (%)
Penicillin	29.4% (10/34)	70.6% (24/34)	0% (0/1)	**100% (1/1)**
Gentamicin	50% (17/34)	50% (17/34)	0% (1/1)	**100% (1/1)**
Erythromycin	70.5% (24/34)	29.5% (10/34)	0% (0/1)	**100% (1/1)**
Amoxicillin+ Clavulanic acid	26.4% (9/34)	73.6% (25/34)	0% (0/1))	**100% (1/1)**
Ceftriaxone	73.5% (25/34)	26.5% (9/34)	100% (1/1)	**0% (0/1))**
Amikacin	32.3%(11/34)	67.7%(23/34)	0% (0/1))	**100% (1/1)**
Doxycyclin	44.1% (15/34)	55.9% (19/34)	100% (1/1)	**0% (0/1)**
Ciprofloxacin	50% (20/34)	50% (17/34)	100% (1/1)	**0% (0/1)**
Cefepime	**58.8% (20/34**	**21.2% (14/34**	**100% (1/1)**	**0% (0/1)**

Table (3-9) showed antibiotics sensitivity agansit Gram negative bacteria isolates from Otitis Media

Isolates / Antibiotics	*Pseudomonas aeruginosa*		*Klebsiella pneumoniae*		*Acinetobacter ursingii*		*Escherichia coli*		*Enterobacter cloacae*		*Proteus mirabilis*	
	Sensitivity (%)	*Resistance (%)*	*Sensitivity (%)*	*Resistance (%)*	*Sensitivity (%)*	*Resistance (%)*	*Sensitivity (%)*	*Resistance (%)*	*Sensitivity (%)*	*Resistance (%)*	*Sensitivity (%)*	*Resistance (%)*
Erythromycin	55.5% (4/7)	44.4% (3/7)	*50% (1/4)*	*75% (3/4)*	50% (1/2)	50% (1/2)	50% (3/6)	50% (3/6)	66.6% (2/3)	33.3% (1/3)	0% (0/1)	100% (1/1)
Amoxicillin Clavulanic Acid	28.5% (2/7)	71.4% (5/7)	*50% (2/4)*	*50% (2/4)*	0% (0/2)	100 % (2/2)	16.6 % (1/6)	83.3 % (5/6)	33.3% (1/3)	66.6% (2/3)	0% (0/1)	100% (1/1)
Ceftriaxone	44.4% (3/7)	55.5% (4/7)	50% (2/4)	50% (2/4)	100% (2/2)	0% (0/2)	66.6 % (4/6)	33.3 % (2/6)	66.6% (2/3)	33.3% (1/3)	100% (1/1)	0% (0/1)
Amikacin	85.7% (6/7)	14.2% (1/7)	75% (3/4)	25% (1/4)	50% (1/2)	50% (1/2)	83.3 % (5/6)	16.6 % (1/6)	100% (3/3)	100% (0/3)	100% (1/1)	0% (0/1)
Gentamicin	71.4% (5/7)	28.5% (2/7)	75% (3/4)	25% (1/4)	*100% (2/2)*	*0% (0/2)*	50% (3/6)	50% (3/6)	33.3% (1/3)	66.6% (2/3)	100% (1/1)	0% (0/1)
Doxycyclin	44.4% (3/7)	55.5% (4/7)	75% (3/4)	25% (1/4)	*0% (0/2)*	*100 % (2/2)*	33.3 % (2/6)	66.6 % (4/6)	33.3% (1/3)	66.6% (2/3)	0% (0/1)	100% (1/1)
Ciprofloxacin	44.4%(3/7)	44.4% (3/7)	50% (24)	50% (2/4)	50% (1/2)	50% (1/2)	66.6 % (4/6)	33.3 % (2/6)	33.3% (1/3)	66.6% (2/3)	100% (1/1)	0% (0/1)
Cefepime	55.5% (4/7)	44.4% (3/7)	50% (2/4)	50% (2/4)	100% (2/2)	0% (0/2)	66.6 % (4/6)	33.3 % (2/6)	66.6% (2/3)	33.3% (1/3)	0% (0/1)	100% (1/1)
Penicillin	14.2% (1/7)	85.7% (6/7)	0% (0/4)	100 % (4/4)	50% (1/2)	50% (1/2)	0% (0/6)	100 % (6/6)	0% (0/3)	100% (3/3)	0% (0/1)	100% (1/1)

3.3.2: Fungal Identification

3.3.2.1: Phenotypic Identification

To study the morphological features of microbial isolates several methods were used, a group of microorganisms have been identified including fungi (molds and yeast). The colony form was adopted as an initial diagnosis, staining smear with (crystal violet) to fungi, observed under oil immersion lens (100X) and Vitek 2 compact system was confirmed the final diagnosis (Appendix, table 3-5). The fungi (molds and yeast), colonies morphology, stain and spore reproduction.

Fungi from mixed isolates were obtained from (15) samples collected (fungi with bacteria). From the results, it was shown that Gram positive bacteria constitutes (45.5%)(41/90) from the total isolates and were considered the predominantan etiological agent of OM compared to Gram negative bacteria which constitute (25.5%) (23/90) while fungi constitute (16.6%) (15/90) As shown in Table (3-5)

The fungi infection higher than others which mixed (fungi with bacterial) because infection was (16.6%).

Aspergillus niger and Yeast (Candida parapsilosis, Malassezia furfur) were isolated from OM in (1.1% and, 11.1%, 4.4%) respectively as shown in Table (3-7). The result also agreement with(Osazuwa et al. 2011) (Juyal et al., 2014) . The result also inagreement with(Osazuwa et al., 2011) found that microorganisms were Aspergillus niger (9.2%) Candida parasilopsis (1.5%).

3.3.2.2: Antifungal sensitivity test

Some antifungal were used to show the effects on different types of fungi isolated from OM. Disc diffusion method was done using different clinically important antifungal.

Cycloheximide is a naturally occurring fungicide produced by the bacterium Streptomyces griseus. Cycloheximide exerts its effects by interfering with the translocation step in protein synthesis (movement of two tRNA molecules and mRNA in relation to the ribosome), thus blocking eukaryotic translational elongation. Cycloheximide is widely used in biomedical research to inhibit protein synthesis in eukaryotic cells studied in vitro(Müller et al., 2000), For this reason, cycloheximide was used in this current study.

A. niger that had either or not been treated with cycloheximide. As expected, cycloheximide blocked secretion of proteins at the periphery of the colony. Unexpectedly, protein release was increased by cycloheximide in the intermediate and central zones of the mycelium when compared to nontreated colonies. Electron microscopy indicated that this is due to partial degradation of the cell wall. This study thus describes spatial release of proteins in A. niger, which is instrumental in understanding how fungi degrade complex substrates in nature(Krijgsheld et al., 2012)

3.4: Immunological Parameters

3.4.1: concentration of human interferon gamma (IFN-γ) in otitis media patients and control

The human interferon gamma (IFN-γ) concentration was measured in serum of patients and control. The results of this test was showed a no significant differences at P ≥0.05 in patients 68.35±2.6 pg/ml comparison with control

61.93±2.5 mg/l can be observed in Table (3-10).

Table (3-10): Concentration of IFN-γ pg/ml in Otitis Media Patients andControl

IFN-γ mg/L	N	Mean ± SD	P. Value of T- test
Patients	**90**	**68.35±2.6**	**0.115**
Control	**30**	**61.93±2.5**	

* significant difference at P≤0.05

** significant difference at P≤0.01

Whenever IFN-concentration in infection gender groups was compared, male otitis media patients had a significant difference (66.983.6 mg/l compared to control). No significant changes were seen in females with otitis media, as evidenced by the patients' 69.434.5 pg/ml compared to the control group's 69.015.7 pg/ml at P.value >0.05, as shown in Table (3-11).

Table (3-11): Concentration of IFN-γ pg/ml in Otitis Media Patients andControl According to Gender Groups

Parameters	Gender	Control	Patient	p-value
		Mean±S.E		
IFN-γ	**Male**	58.39±6.9	66.98±3.6	**0.043***
	Female	69.01±5.7	69.43±4.5	**0.674**
p-value		0.142		

* significant difference at P≤0.05

** significant difference at P≤0.01

The significant IFN-γ concentration increase appears high level in bacterial infection groups at the mean ± SD 64.68±3.4 [a] pg/ml in comparison with control

group at the mean ± SD 61.93±2.5 [a] pg/ml, while no significant differences the mean level at the concentration in no growth (unknown causative agent infection) 70.04±5.2 [ab] pg/ml and fungal infection 75.03±5.5 [bc] pg/ml comparison with control 61.93±2.5 [a] pg/ml can be observed in Table (3-12).

Table (3-12): Concentration of IFN-γ pg/ml in Otitis Media Patients andControl According to Causative Agent

IFN-γ mg/L	causative agent	Mean ± SD
Patient	Bacterial infection	64.68±3.4 [a]
	Fungal infection	75.03±5.5 [bc]
	no growth	70.04±5.2 [ab]
Control		61.93±2.5 [a]

Different letters refer on significant differences.

This result might show that the infection is mostly at acute stage due to increase of the IFN-γ levels. This result might be show that the fungi causes of otitis media in more induced of IFN-γ than other causes such as bacterial and unknown causes. This result agree with (Ahn et al., 2016).

Interferon-γ (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) activate microbial killing by polymorphonuclear leukocytes (PMNs), and IFN-γ, GM-CSF and macrophage colony-stimulating factor (M-CSF) increase killing by monocytes and macrophages(Abzug and Walsh, 2004).

Use of these immune modulators may selectively promote a T_H1 response, more likely to induce protective immunity against fungal pathogens(Clemons and Stevens, 2001, Roilides and Farmaki, 2001).

3.4.2: Concentration of human Interleukin 10 (IL-10) in otitis media patients and control

The human Interleukin 10 (IL-10) concentration was measured in serum of patients and control. The results of this test was showed a high significant differences at P <0.05 in patients 28.59±0.9 pg/ml comparison with control 43.07±1.9 pg/ml can be observed in Table (3-13).

Table (3-13): Concentration of IL-10 pg/ml in Otitis Media Patients and Control.

IL-10 mg/L	N	Mean ± SD	P. Value of T- test
Patients	**90**	**28.59±0.9**	≤0.0001**
Control	**30**	**43.07±1.9**	

* significant difference at P≤0.05

** significant difference at P≤0.01

When compared IL-10 concentration in infection gender groups a significant difference was showed in male and female in otitis media as in the patients 28.08±1.7 pg/ml and 29.23±1.2 pg/ml respectively comparison with control 41.66±5.4 pg/ml and 45.89±3.9 pg/ml respectively at P.value <0.05 can be observed in Table (3-14).

Table (3-14): Concentration of IL-10 mg/I in Otitis Media Patients andControl According to Gender Groups

Parameters	Gender	Control	Patient	p-value
		Mean±S.E		
IL-10	**Male**	41.66±5.4	28.08±1.7	**0.001****
	Female	45.89±3.9	29.23±1.2	**0.002****
p-value		0.567		

* significant difference at P≤0.05 ,

** significant difference at P≤0.01

The significant IL-10 concentration increase appears high level in bacterial infection groups at the mean ± SD 29.90±1.6[a] pg/ml then fungal infection groups at the mean ± SD 27.82±1.8[a] pg/ml and no growth (unknown causative agent infection) groups at the mean ± SD 27.12±1.5[a] pg/ml in comparison with control group at the mean ± SD 43.07±1.9[b] pg/ml, can be observed in Table (3-15).

Table (3-15): Concentration of IL-10 mg/I in Otitis Media Patients andControl According to Causative Agent

IL-10 mg/L	causative agent	Mean ± SD
Patient	Bacterial infection	29.90±1.6[a]
	Fungal infection	27.82±1.8[a]
	no growth	27.12±1.5[a]
Control		43.07±1.9[b]

Different letters refer on significant differences.

This result might be show that all causative agent groups is more induced immune responses in bacterial infection were the IL-10 is high level in serum, this result agree with *(Peñaloza et al., 2016)* content its IL-10 is frequently secreted during acute bacterial infections and has been described to play a key role in infection resolution, although its effects can significantly vary depending on the infecting bacterium, while the production of IL-10 might favor host survival in some cases, it may also result harmful for the host in other circumstances, as it can prevent appropriate bacterial clearance.

This result might show the humoral immunity activity (Th2) as which induce secretion IL-10 as defense response of immune system against pathogens. It is agree with the studies (Dhanda *et al*., 2013) , and from cytokines identified in

otitis media, immunoregulatory cytokine [interleukin-10 (IL-10)] is mediators of the immune system, as a crucial molecular regulator , IL-10 is involved in regulation of the middle ear inflammatory response induced by bacterial infection ((Smirnova et al., 2004).

3.4.3: Concentration of Human Cytotoxic T Lymphocyte Associated Antigen 4 (CTLA-4) in otitis media patients and control

As illustrated in Table (3-16) that show the mean of Human Cytotoxic T Lymphocyte Associated Antigen 4 (CTLA-4) concentration in patients 42.99±1.4 mg/L was a significant increased P <0.05 compared with the control group 34.03±1.7 ng/ml.

Table (3-16): Concentration of CTLA-4 mg/L in Otitis Media Patients and Control.

CTLA-4 mg/L	N	Mean ± SD	P. Value of T- test
Patients	**90**	42.99±1.4	0.001**
Control	**30**	34.03±1.7	

* significant difference at P≤0.05

** significant difference at P≤0.01

When compared CTLA-4 concentration in infection gender groups a significant difference was showed in male and female in otitis media as in the patients 41.85±1.8 ng/ml and 44.77±1.5 ng/ml respectively comparison with control 33.45±2.3 ng/ml and 34.42±2.1 mg/l respectively at P.value <0.05 can be observed in Table (3-17).

Table (3-17): Concentration of CTLA-4 ng/ml in Otitis Media Patients and Control According to Gender Groups.

Parameters	Gender	Control	Patient	p-value
		Mean±S.E		
CTLA-4	**Male**	33.45±2.3	41.85±1.8	**0.036***
	Female	34.42±2.1	44.77±1.5	**0.026***
p-value		0.397		

* significant difference at P≤0.05

** significant difference at P≤0.01

The result of CTLA-4 of a present study shows that a significant increased at P<0.05 fungal causes group 43.79±2.2 ^{b} ng/ml while less significant increase in bacterial causes groups a t the mean ± SD 42.48±1.8 ^{b} mg/l in comparison with control group 34.03±1.7 ^{a} ng/ml. can be observed in Table (3-18).

Table (3-18): Concentration of CTLA-4 mg/I in Otitis Media Patients and Control According to Causative Agent.

CTLA-4 mg/L	causative agent	Mean ± SD
Patient	**Bacterial infection**	42.48±1.8 ^{b}
	Fungal infection	43.79±2.2 ^{b}
Control		34.03±1.7 ^{a}

Different letters refer on significant differences.

The result of the current work indicated that is difference of the CTLA-4 concentration in the bacterial infection Otitis Media patients and bacterial

infection OM patients. It means that a bacterial infection has a stronger effect on suppressing immunity. This result agree with(Lin et al., 2014)

CTLA-4, a surface receptor of immunoglobulins (Lecocq *et al.*,2021), T-cell activation inhibitor Co-receptor cytotoxic T-cell lymphocyte antigen-4 (CTLA-4; CD 152) is a key T-cell proliferation and expansion inhibitor (van Beers *et al.*,2021), it has damping effect on the activation mechanism and terminates T-cell responses. As such, T-cell tolerance and autoimmunity are necessary to regulate (Schneider *et al.,2014*).

CTLA-4, the first scientifically targeted immune control point receptor, is found exclusively on T cells where the amplitude of the early stage of T cell activation is primarily controlled (Pardoll et al.,2014). CTLA-4, a surface receptive immunoglobulin cell, is a T-cell activation inhibitor. (Dovedi *et al.*,2021). It is expressed predominantly on naive T cells after activation (Thorsen *et al.*,2021).

CTLA-4 is a high affinity CD28 homologue for B7- 1/2. Although the interaction of the CD 28: B7- 1/2 serves as a co-simulator for T-cell propagation and activation, CTLA-4: B7- 1/2 binding, it is also a co-inhibitory signal to stop early T-cell activation, leading to inhibition of T-cell stimulation (Mpakali *et al.*,2021; Vella *et al.*,2021).

T-cell activation relies not only on the T-cell receptor (TCR) binding to the antigen provided by the antigen presenting cell (APC), but also with the existence of the costimulatory second signal, usually by binding the CD28 displayed on the T-cell to the CD80/86 located on the APC, the loss of this secondary signal will lead to a T-cell being identified the presented peptide as a "self-antigen" or developing antigen-tolerance (Mir *et al* .,2021).

TCR signaling immediately up-regulates CTLA-4 expression on the cell surface, reaching peak expression 2 to 3 days' post stimulation (Lafleur *et al.*,2021), provides a negative feedback loop upon activation of T-cells. CTLA-4 inside the intracellular vesicles is also rapidly transferred to the immunological synapse after T-cell activation (Willsmore *et al.*,2021; Felce *et al* .,2021).

CTLA-4 is stabilized with the CD80/CD86 binding in the immune synapse, allowing the CD28 binding to be collected and inhibited. CTLA-4 restricts CD28 signalization downstream, inhibits the pathway PI3K and AKT (de Araújo *et al* .,2021). CTLA-4 also removes CD28 ligands CD80/86 from adjacent cells by trans endocytosis in *vivo*, like APCs, besides inhibiting T-cell activation (Wu *et al* .,2021).

Physiologically, CTLA-4 is known mainly to play a modulatory role in T-cell priming in local secondary lymphoid organs, by suppressing T-cell activation and prevent the progression of T-cells (Adamo *et al* .,2021).

CTLA-4 was among the first and most thoroughly studied immune system co-inhibitor receptors (Okoye *et al*., 2020). Given the significance of CTLA-4 for autoimmunity and anti-tumor immunotherapy, the precise pathways responsible for its function are unclear. Much controversy has centered on whether CTLA-4 inhibits T cell response by extrinsic or-intrinsic cell mechanisms (Guo *et al.*,2019). Cell-intrinsic mechanisms will represent the direct effects of the co-receptor on the expression cell, whereas cell-extrinsic effects are linked to the modulation of activity by the distal cell or cytokine. Both pathways have been involved in the *in vivo activity* of CTLA-4 (Lindau., 2020; Liu.,2020). Susceptibility of those pate

Thus, upon the results of the present work, the increasing level of CTLA-4 (immune suppressor factor) in otitis media patients as cowpared to apparently healthy control could be interpreted the high susceptibility of those patients to microbial infections.

CONCLUSIONS

and

RECOMMENDATIONS

Conclusions

1- Otitis media is a common infection in both children and adults.

2- Children under 10 years of age were the predominant group affected by otitis media accounting for 16.6 %.

3- There is a slight male predilection in the current study regarding the acquisition of otitis media.

4- *Staphalococcus aureus* and *Pseudomonas aeruginosa* were the predominant pathogens of discharging ear followed by *S. epidermidis, Escherichia coli Klebisella pneumonia and Enterobacter cloacae,* while *Candida parapsilosis* was the dominant among fungi.

5- In this study were increasingly becoming more resistance to the commonly used antibiotic like Amoxicillin + Clavulanic acid, Penicillin and Doxycycline, while highly sensitive to Ceftriaxone and Erythromycin in Gram negative bacterial while in Gram positive bacterial is, Amikacin and Gentamicin either fungal increase resistance to Cycloheximide in Candida parapsilosis and Malassezia furfur while highly sensitive in Aspergillus niger.

6- Signification increasing of immunological parameters was shown in patients with control especially IFN-γ and CTLA-4 while in the interleukin-10 is a decrease in concentration to patient's serum.

Recommendations

1- The curried study suggests of the important culture and antibiotics sensitivity test for every patient with otitis media to describe suitable antibiotics according to results of culture.

2- Appropriate and early diagnosis with optimal management for the pediatric patients under 15 years of age with acute otitis media to reduce the risk of conversion to recurrent or chronic disease.

3- More studies for other microbial infection such as viruses, parasites, *Chlamydia* and *mycoplasma*.

4- Farther studies for other immunity parameters.

5- Research on effective otitis media prevention, screening and treatment strategies and evaluation of programs should be supported and used to guide future investment. This should include collection of accurate prevalence data through surveillance activities.

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Appendices

Appendix (1): Questionnaire sheet sample for patients in this study.

PATIENT QUESTIONNAIRE

General Data

Sample ID		Name					
Gender	Male		Female				
Age							
Newborn (0–4 weeks); Infant (4 weeks – 1 year); Toddler (12 months-24 months); Preschooler (2–5 years); School-aged child (6–12 years); Adolescent (13–19).							
Residency Site	Urban		Rural				
Type of Inflammation	AOM		OME		CSOM		
Recurrent Inflammation	Yes		No				
Tonsillectomy	Yes		No				
Heredity Factor	Yes		No				
Other Chronic Disease							
Antibacterial Treatment		Antiviral Treatment					
Blood Group	A^+		B^+		AB^+	O^+	
	A^-		B^-		AB^-	O^-	
Date of Inflammation	DD		MM		YYYY		
Date of Sampling	DD		MM		YYYY		

Clinical Features

Pain	Malaise	Fever	Reduced Hearing	Tenderness of Skin	Purulent Discharge	Diarrhea	URTI	Cough	Nasal Discharge	Irritability

Appendix (2): Questionnaire sheet sample for control subjects in this study.

CONTROL QUESTIONNAIRE

General Data

Sample ID		Name							
Gender	Male			Female					
Age									
Newborn (0–4 weeks); Infant (4 weeks – 1 year); Toddler (12 months-24 months); Preschooler (2–5 years); School-aged child (6–12 years); Adolescent (13–19).									
Residency Site	Urban			Rural					
Tonsillectomy	Yes			No					
Other Chronic Disease									
Antibacterial Treatment			Antiviral Treatment						
Blood Group	A^+		B^+		AB^+		O^+		
	A^-		B^-		AB^-		O^-		
Date of Sampling	DD		MM		YYYY				

Appendix (3): Vitek 2 system result showed 11 samples of *Malassezia furfur*.

HAMMURABI LAB.

bioMérieux Customer: Microbiology Chart Report Printed January 24, 2022 6:51:40 PM AST

Patient Name: Said, Anas
Location:
Lab ID: 57

Patient ID: 57
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Malassezia furfur

Source: Otitis **Collected:**

Comments:	

Identification Information	**Analysis Time:**	17.78 hours	**Status:**	Final
Selected Organism	95% Probability	**Malassezia furfur**		
	Bionumber:	0000000020000000		
ID Analysis Messages				

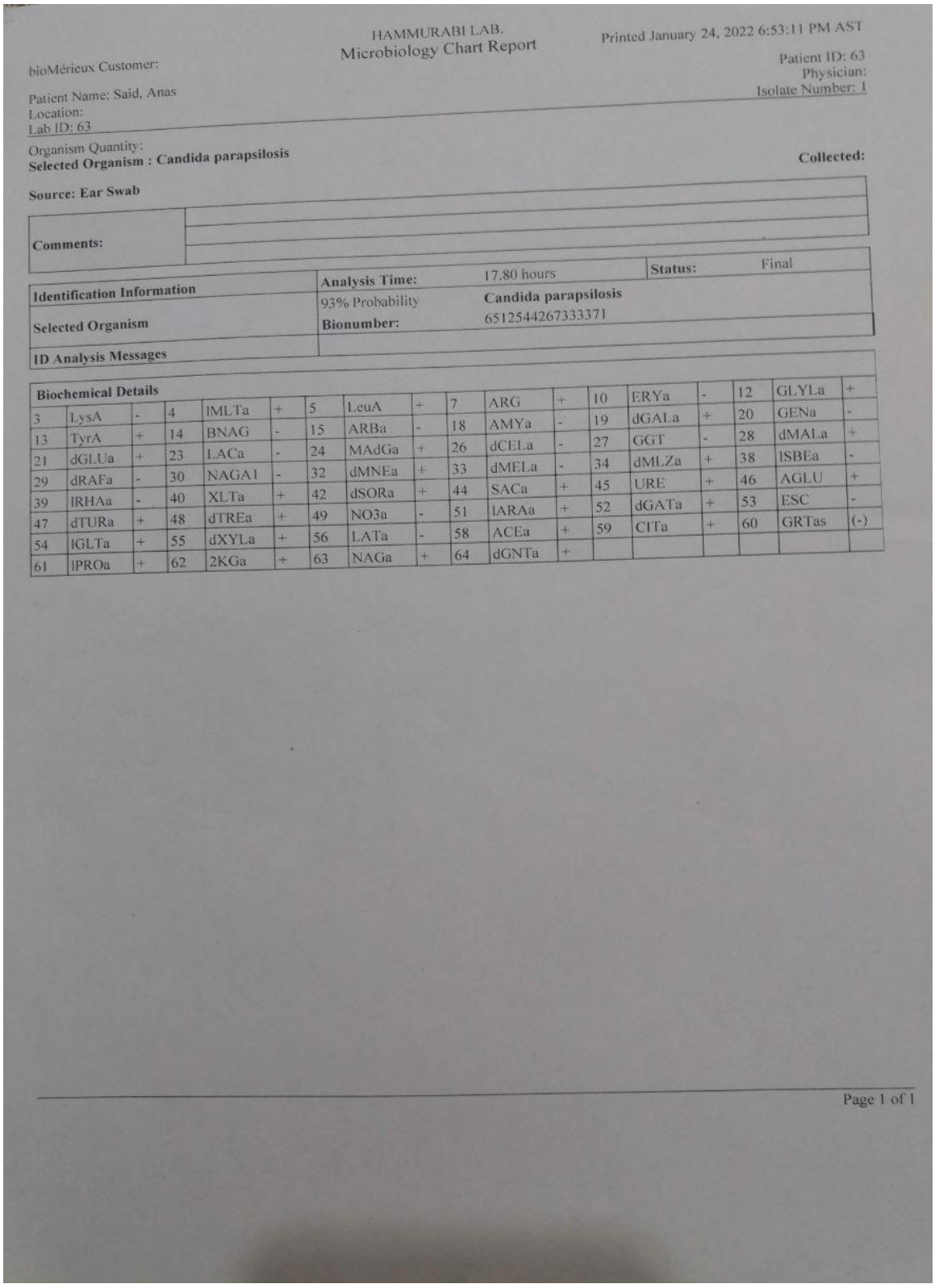
Susceptibility Information	**Analysis Time:**	36.12 hours	**Status:**	Final

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Fluconazole			Micafungin		
Voriconazole			Amphotericin B		
Caspofungin			Flucytosine		

AES Findings	
Confidence:	Unknown

Page 1 of 1

Appendix (4): Vitek 2 system result showed 51 samples of *Candida parapsilosis.*



HAMMURABI LAB.
Microbiology Chart Report

Printed January 24, 2022 6:53:11 PM AST

bioMérieux Customer:

Patient ID: 63
Physician:
Isolate Number: 1

Patient Name: Said, Anas
Location:
Lab ID: 63

Organism Quantity:
Selected Organism : Candida parapsilosis

Collected:

Source: Ear Swab

Comments:	

Identification Information	**Analysis Time:**	17.80 hours	**Status:**	Final
	93% Probability	**Candida parapsilosis**		
Selected Organism	**Bionumber:**	6512544267333371		
ID Analysis Messages				

Biochemical Details

3	LysA	-	4	lMLTa	+	5	LeuA	+	7	ARG	+	10	ERYa	-	12	GLYLa	+
13	TyrA	+	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGA1	-	32	dMNEa	+	33	dMELa	-	34	dMLZa	+	38	lSBEa	-
39	lRHAa	-	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	+	46	AGLU	+
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	lARAa	+	52	dGATa	+	53	ESC	-
54	lGLTa	+	55	dXYLa	+	56	LATa	-	58	ACEa	+	59	CITa	+	60	GRTas	(-)
61	lPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	+						

Page 1 of 1

Appendix (5): Vitek 2 system result showed 1 samples of *Klebsiella pneumoniae*.

bioMérieux Customer: Microbiology Chart Report Printed Dec 22, 2021 15:32 CST

Patient Name:
Location:
Lab ID: 2212215
Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Klebsiella pneumoniae

Source:
Collected:

Comments:	

Identification Information	Analysis Time: 6.98 hours	Status: Final
Selected Organism	Klebsiella pneumoniae	
ID Analysis Messages		

Susceptibility Information	Analysis Time: 10.23 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
+Amoxicillin		R	Amikacin	<= 2	S
+Ampicillin/Sulbactam			Gentamicin	<= 1	S
Ticarcillin	<= 8*	*R	Tobramycin	<= 1	S
Ticarcillin/Clavulanic Acid	<= 8	S	+Nalidixic Acid		
Piperacillin	>= 128	R	Ciprofloxacin	<= 0.25	S
+Piperacillin/Sulbactam			+Enrofloxacin		
Piperacillin/Tazobactam	<= 4	S	+Fleroxacin		
+Cefalexin		R	+Levofloxacin		S
+Cefazolin		R	+Moxifloxacin		S
+Cefoxitin			+Norfloxacin		
+Cefixime		R	+Ofloxacin		S
+Cefpodoxime		R	Pefloxacin		
+Cefotaxime		R	+Tosufloxacin		
Ceftazidime	4	*R	+Doxycycline		S
+Ceftizoxime		R	Minocycline	<= 1	S
+Ceftriaxone		R	+Tetracycline		S
+Ceftazidime/Avibactam			Colistin		
Cefepime	<= 1	*R	Rifampicin		
Aztreonam	>= 64	R	+Trimethoprim		
Imipenem	<= 0.25	S	Trimethoprim/Sulfamethoxazole	<= 20	S
Meropenem	<= 0.25	S			

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Consistent with correction

Appendix (6): Vitek 2 system result showed 26 samples of *Escherichia coli*.

bioMérieux Customer:

Microbiology Chart Report

Printed Dec 22, 2021 19:30 CST

Patient Name:
Location:
Lab ID: 1812211

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Escherichia coli

Source:

Collected:

Comments:	

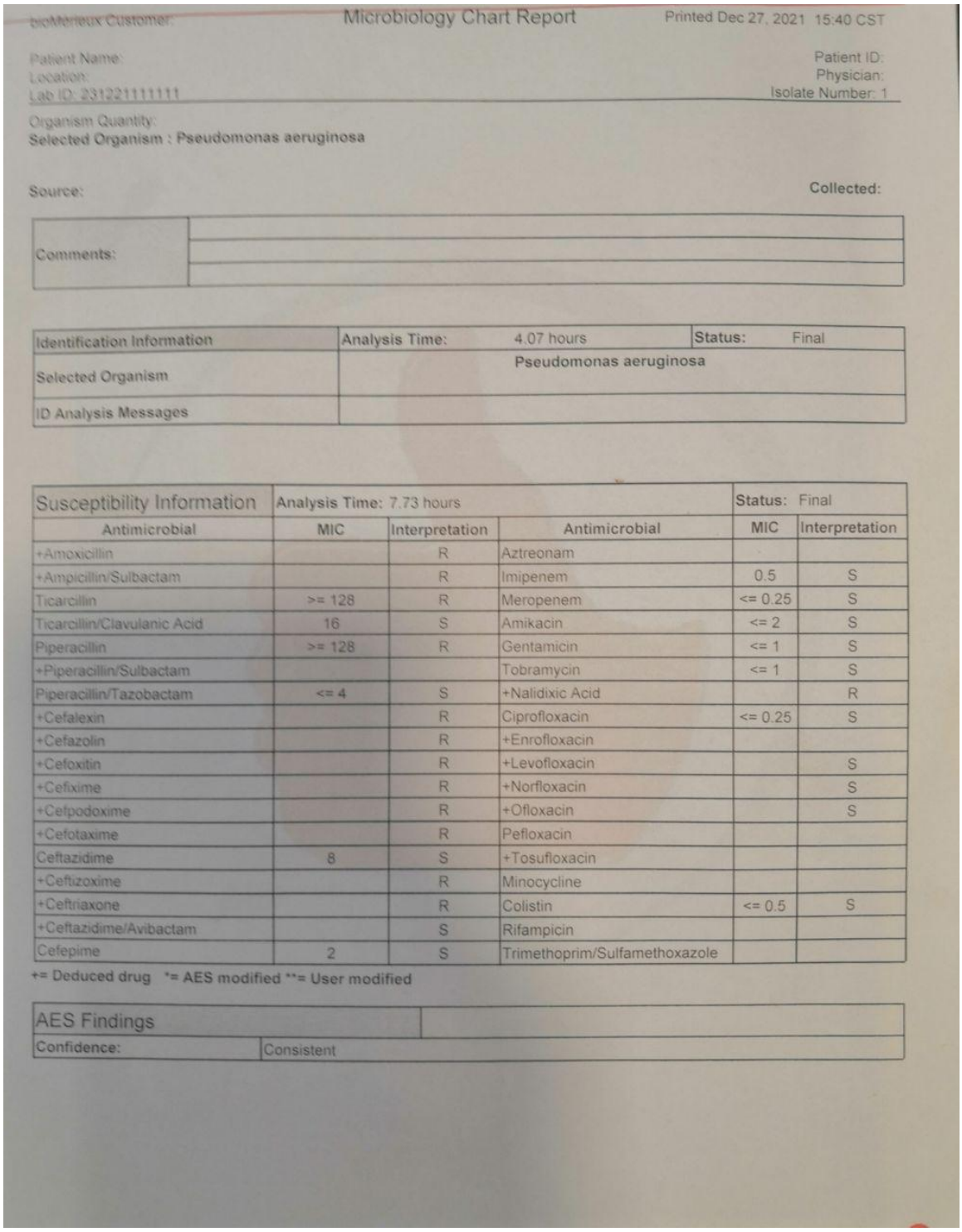
Identification Information	Analysis Time:	4.85 hours	Status:	Final
Selected Organism		Escherichia coli		
ID Analysis Messages				

Susceptibility Information	Analysis Time: 8.75 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretatio
+Amoxicillin		R	Amikacin	<= 2	S
+Ampicillin/Sulbactam		R	Gentamicin	<= 1	S
Ticarcillin	32	*R	Tobramycin	<= 1	S
Ticarcillin/Clavulanic Acid	<= 8	*R	+Nalidixic Acid		R
Piperacillin	8	*R	Ciprofloxacin	0.5	S
+Piperacillin/Sulbactam		R	+Enrofloxacin		
Piperacillin/Tazobactam	TRM		+Fleroxacin		R
+Cefalexin		R	+Levofloxacin		S
+Cefazolin		R	+Moxifloxacin		S
+Cefoxitin		R	+Norfloxacin		R
+Cefixime		R	+Ofloxacin		S
+Cefpodoxime		R	Pefloxacin		
Ceftazidime	2	S	+Tosufloxacin		
+Ceftizoxime			+Doxycycline		
+Ceftriaxone		R	Minocycline	4	S
+Ceftazidime/Avibactam		S	+Tetracycline		
Cefepime	<= 1	S	Colistin		
Aztreonam	16	R	Rifampicin		
Imipenem	TRM		+Trimethoprim		
Meropenem	<= 0.25	S	Trimethoprim/Sulfamethoxazole	<= 20	S

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Consistent

Appendix (7): Vitek 2 system result showed 28 samples of *Pseudomonas auroginosa*.



bioMérieux Customer: Microbiology Chart Report Printed Dec 27, 2021 15:40 CST

Patient Name: Patient ID:
Location: Physician:
Lab ID: 231221111111 Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: Collected:

Comments:	

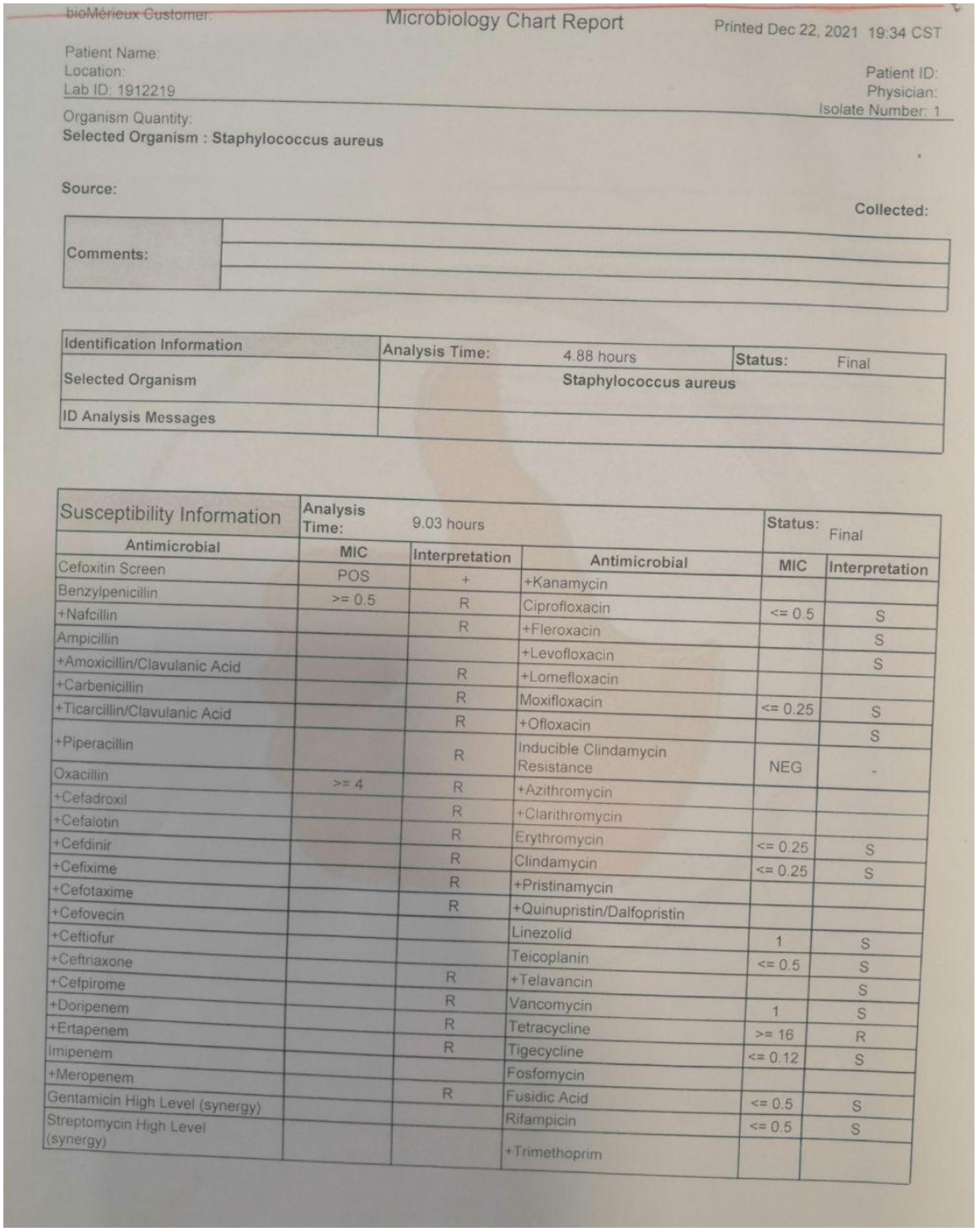
Identification Information	Analysis Time:	4.07 hours	Status:	Final
Selected Organism	Pseudomonas aeruginosa			
ID Analysis Messages				

Susceptibility Information	Analysis Time: 7.73 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
+Amoxicillin		R	Aztreonam		
+Ampicillin/Sulbactam		R	Imipenem	0.5	S
Ticarcillin	>= 128	R	Meropenem	<= 0.25	S
Ticarcillin/Clavulanic Acid	16	S	Amikacin	<= 2	S
Piperacillin	>= 128	R	Gentamicin	<= 1	S
+Piperacillin/Sulbactam			Tobramycin	<= 1	S
Piperacillin/Tazobactam	<= 4	S	+Nalidixic Acid		R
+Cefalexin		R	Ciprofloxacin	<= 0.25	S
+Cefazolin		R	+Enrofloxacin		
+Cefoxitin		R	+Levofloxacin		S
+Cefixime		R	+Norfloxacin		S
+Cefpodoxime		R	+Ofloxacin		S
+Cefotaxime		R	Pefloxacin		
Ceftazidime	8	S	+Tosufloxacin		
+Ceftizoxime		R	Minocycline		
+Ceftriaxone		R	Colistin	<= 0.5	S
+Ceftazidime/Avibactam		S	Rifampicin		
Cefepime	2	S	Trimethoprim/Sulfamethoxazole		

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Consistent

Appendix (8): Vitek 2 system result showed 34 samples of *Staphylococcus auresus.*



bioMérieux Customer: Microbiology Chart Report Printed Dec 22, 2021 19:34 CST

Patient Name:
Location:
Lab ID: 1912219
Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Staphylococcus aureus

Source:

Collected:

Comments:	

Identification Information	Analysis Time: 4.88 hours	Status: Final
Selected Organism	Staphylococcus aureus	
ID Analysis Messages		

Susceptibility Information	Analysis Time: 9.03 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Cefoxitin Screen	POS	+	+Kanamycin		
Benzylpenicillin	>= 0.5	R	Ciprofloxacin	<= 0.5	S
+Nafcillin		R	+Fleroxacin		S
Ampicillin			+Levofloxacin		S
+Amoxicillin/Clavulanic Acid		R	+Lomefloxacin		
+Carbenicillin		R	Moxifloxacin	<= 0.25	S
+Ticarcillin/Clavulanic Acid		R	+Ofloxacin		S
+Piperacillin		R	Inducible Clindamycin Resistance	NEG	-
Oxacillin	>= 4	R	+Azithromycin		
+Cefadroxil		R	+Clarithromycin		
+Cefalotin		R	Erythromycin	<= 0.25	S
+Cefdinir		R	Clindamycin	<= 0.25	S
+Cefixime		R	+Pristinamycin		
+Cefotaxime		R	+Quinupristin/Dalfopristin		
+Cefovecin			Linezolid	1	S
+Ceftiofur			Teicoplanin	<= 0.5	S
+Ceftriaxone		R	+Telavancin		S
+Cefpirome		R	Vancomycin	1	S
+Doripenem		R	Tetracycline	>= 16	R
+Ertapenem		R	Tigecycline	<= 0.12	S
Imipenem			Fosfomycin		
+Meropenem		R	Fusidic Acid	<= 0.5	S
Gentamicin High Level (synergy)			Rifampicin	<= 0.5	S
Streptomycin High Level (synergy)			+Trimethoprim		

Appendix (9): Vitek 2 system result showed 88 samples of *Streptococcus pyogenes*.

bioMérieux Customer:
System #:

Laboratory Report

Printed Dec 27, 2021 15:03 CST
Printed by: Labadmin

Patient Name:
Isolate: 26122120-1 (Qualified)

Patient ID:

Card Type: AST-ST03 Bar Code: 5421616503706339 Testing Instrument: 00000A726B5A (AL-NUKHBA LAB)
Setup Technologist: Laboratory Administrator(Labadmin)

Organism Quantity: **Selected Organism: Streptococcus pyogenes**

Comments:	

Identification Information				
Organism Origin	Technologist			
Selected Organism	Streptococcus pyogenes			
	Entered:	Dec 26, 2021 19:02 CST	**By:**	Labadmin

Analysis Messages:

High level resistance to gentamicin (MIC of > 128 mg/L), is generally caused by the production of a bifunctional APH(2')-AAC(6) enzyme that determines loss of synergism of all aminoglycosides (except streptomycin and arbekacin) with b-lactams and glycopeptides irrespective of MIC values.

Susceptibility Information	Card:	AST-ST03	Lot Number:	5421616503	Expires:	Apr 30, 2022 13:00 CDT
	Completed:	Dec 26, 2021 22:14 CST	Status:	Final	Analysis Time:	11.40 hours

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Benzylpenicillin	1		Clindamycin	<= 0.25	S
Ampicillin	2		Linezolid	<= 2	S
Cefotaxime	1		Teicoplanin		
Ceftriaxone	1		Vancomycin	1	S
Gentamicin			Tetracycline	>= 16	R
Levofloxacin	2	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.25	S	Chloramphenicol	2	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	2	R	Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug *= AES modified **= User modified

AES Findings:	**Last Modified:** Jan 7, 2021 15:14 CST	**Parameter Set:** Global CLSI-based+Phenotypic 2019
Confidence Level:	Inconsistent	

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline: Global CLSI-based 2019
AES Parameter Set Name: Global CLSI-based+Phenotypic 2019

Therapeutic Interpretation Guideline: PHENOTYPIC 2019
AES Parameter Last Modified: Jan 7, 2021 15:14 CST

Appendix (11): Vitek 2 system result showed 41 samples of *Enterobacter cloacae*.

bioMerieux Customer: Microbiology Chart Report Printed Dec 26, 2021 19:18 CST

Patient Name: Patient ID:
Location: Physician:
Lab ID: 231221111111 Isolate Number: 1

Organism Quantity:
Selected Organism : Enterobacter cloacae ssp dissolvens

Source: Collected:

Comments:	

Identification Information	Analysis Time: 4.07 hours	Status: Final
Selected Organism	Enterobacter cloacae ssp dissolvens	
ID Analysis Messages		

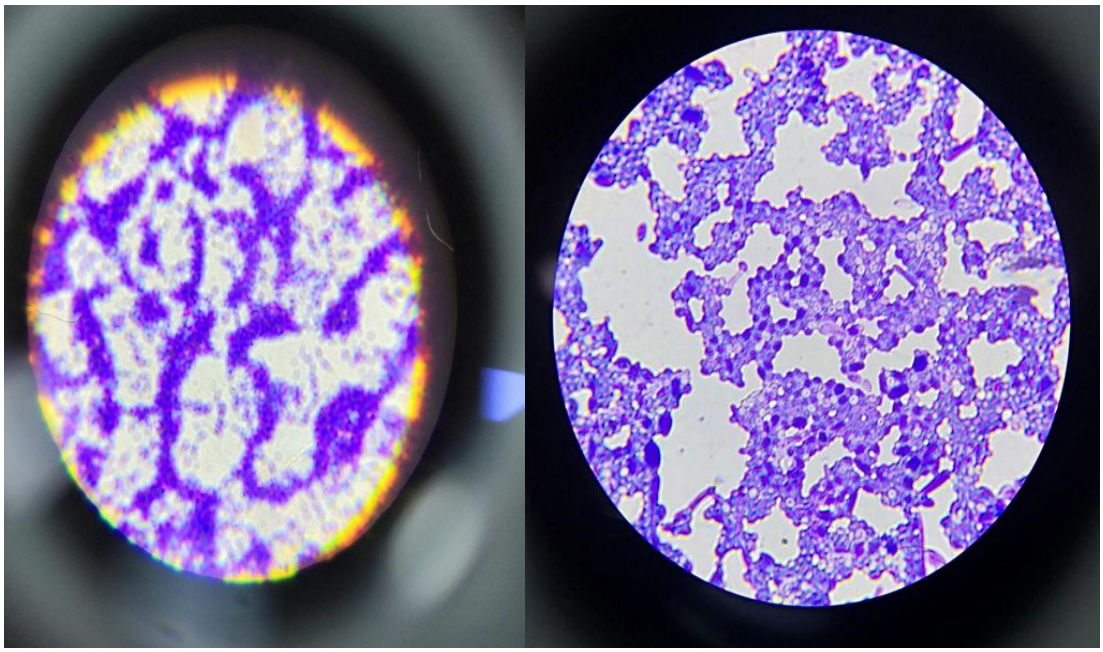
Susceptibility Information	Analysis Time: 8.95 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
+Amoxicillin		R	Amikacin	<= 2	S
+Ampicillin/Sulbactam		R	Gentamicin	<= 1	S
Ticarcillin	>= 128	R	Tobramycin	<= 1	S
Ticarcillin/Clavulanic Acid	16	S	+Nalidixic Acid		
Piperacillin	>= 128	R	Ciprofloxacin	<= 0.25	S
+Piperacillin/Sulbactam			+Enrofloxacin		
Piperacillin/Tazobactam	<= 4	S	+Fleroxacin		
+Cefalexin		R	+Levofloxacin		S
+Cefazolin		R	+Moxifloxacin		S
+Cefoxitin		R	+Norfloxacin		
+Cefixime		R	+Ofloxacin		S
+Cefpodoxime		R	Pefloxacin		
+Cefotaxime		R	+Tosufloxacin		
Ceftazidime	8	*R	+Doxycycline		R
+Ceftizoxime		R	Minocycline	>= 16	R
+Ceftriaxone		R	+Tetracycline		R
+Ceftazidime/Avibactam		S	Colistin		
Cefepime	2	*R	Rifampicin		
Aztreonam	16	R	+Trimethoprim		R
Imipenem	<= 0.25	S	Trimethoprim/Sulfamethoxazole	>= 320	R
Meropenem	<= 0.25	S			

+= Deduced drug *= AES modified **= User modified

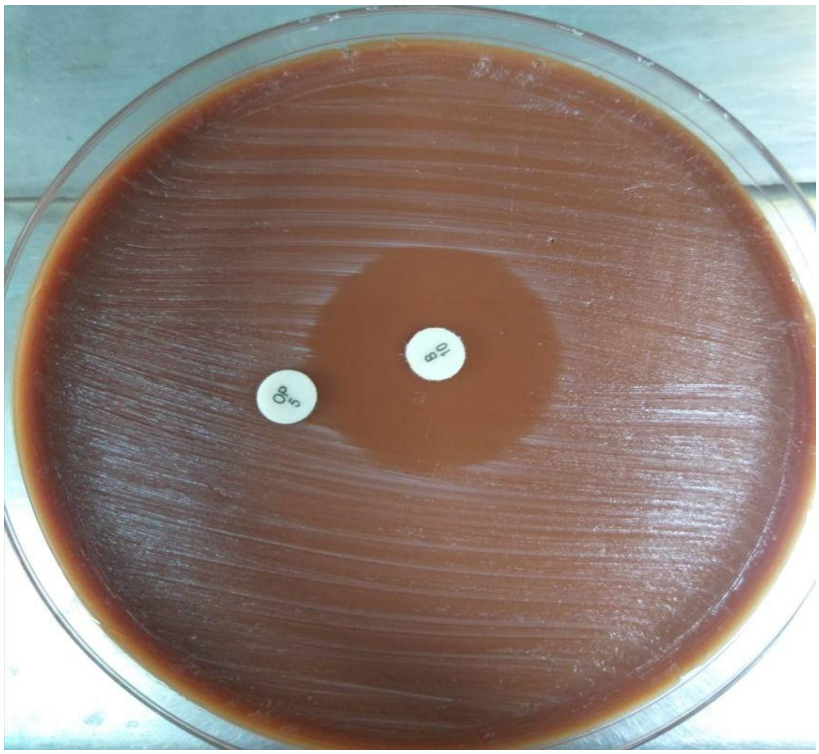
AES Findings	
Confidence:	Consistent

Appendix (12): Growth *Escherichia coli* of on MacConkey agar

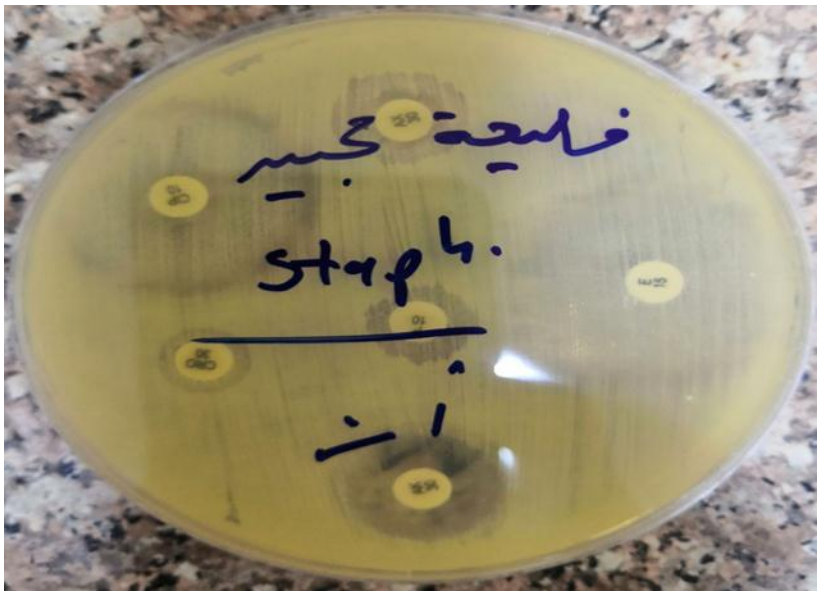
Appendix (13): Growth *Malassezia furfur* on Sabroud agar



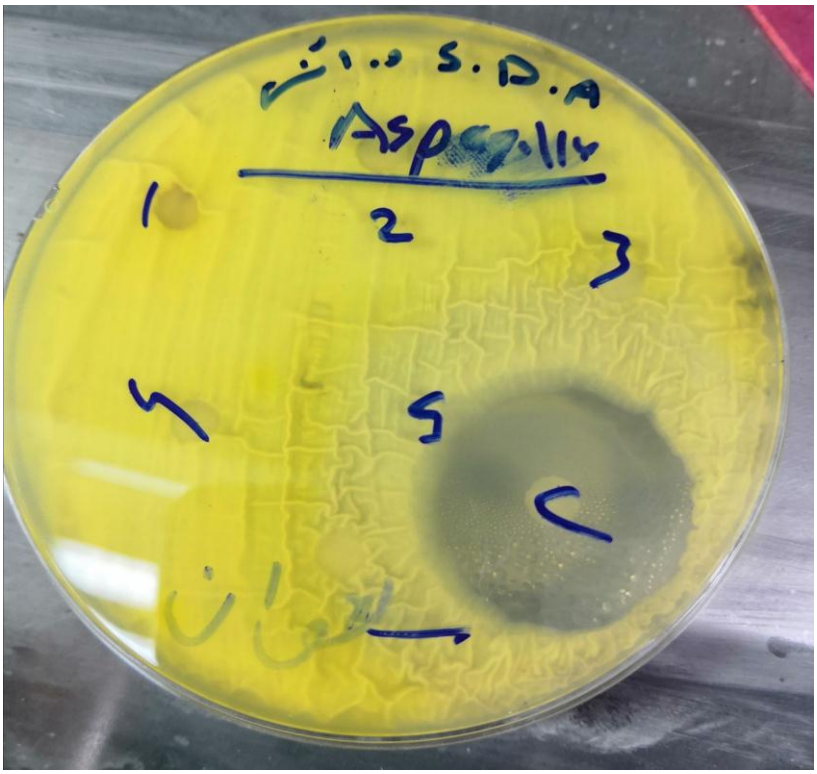
Appendix (14): Staphylococcus aureus under light microscope by Gram stain



Appendix(15): Zone appearance of AST for Streptococcus pyogenes on Joklet agar



Appendix(16): Zone appearance of AST for Staphylococcus aureus on MHA



Appendix (17): Zone appearance of AST for Aspergillus niger on Sabroud agar

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل

كلية الطب

دراسة بعض الواسمات المناعية وقابلية مضادات الميكروبات على العزلات السريرية من مرض التهاب الاذن الوسطى

رسالة مقدمة الى

مجلس كلية الطب / جامعة بابل

هي جزء من متطلبات نيل درجة الماجستير في علوم الاحياء المجهرية الطبية

من قبل

أنس رزاق محسن العوادي

بكلوريوس / علوم حياة / جامعة بابل / 2006

بأشراف

أ.د محمد عبد كاظم السعدي

د. عدي حسين كاظم

1443هـ

2022

الخلاصة

يعد التهاب الأذن الوسطى مشكلة صحية للأطفال وكذلك البالغين والسبب الرئيسي لفقدان السمع والصمم الكامل وشلل الوجه وخراج المخ والتهاب الأذن الداخلية والتهاب الغشاء المبطن.

تضمنت الدراسة جمع عينات (مسحات الأذن الوسطى) وثقافتها المخبرية من (ن = 90) مريضاً، 48 منهم من الذكور و42 من الإناث المصابين بعدوى الأذن الوسطى بمستشفى الحلة التعليمي (وحدة الأنف والأذن والحنجرة) ومستشفى الإمام الصادق (الأنف والأذن والحنجرة). الوحدة) في مدينة بابل خلال الفترة ما بين (أكتوبر 2021 إلى فبراير 2022) المعاناة من التهاب الأذن الوسطى ((OM، لفحص مسببات الأمراض الجرثومية، تم جمع عينات الدم (90) و (30) عينة من الدم على ما يبدو لفصل عينات المصل. بالنسبة للسيتوكين (IFN-γ، IL-10)وتقدير CTLA، تم تحديد العزلات وفقًا لاختبارات ثقافية وكيميائية حيوية، تم تأكيدها بواسطة نظام VITEK 2 المضغوط وطريقة التشخيص الجزيئي PCR، وكانت النتائج الإيجابية للثقافة المختبرية (64) أظهر المرضى مقابل (26) مريضا ثقافة سلبية.

تم توزيع المرضى على ثماني مجموعات حسب العمر، 1 - 9 سنوات (15) مريضا، 10 - 19 سنة (12) مريضا، 20 - 29 سنة (14) مريضا، 30 - 39 سنة (11) مريضا، 40 - 49 عامًا (10) مريضا، 50-59 عامًا (9) مريضا، 60-69 عامًا (9) مريضا و70-80 عامًا (10) مريضا، أظهرت النتائج أن أعلى معدل للإصابة كان في الفئة العمرية الأولى (1-9 سنوات) بنسبة (16.7٪).

وجد في هذه الدراسة أن أكثر الأمراض المسببة للمرض يتم تشخيصها على النحو التالي:

- العوامل البكتيرية 64 (71.1٪) من 90 عينة، أكثر أنواع المعزولات البكتيرية شيوعاً هي:

الخلاصة

- عزلات بكتيرية صبغة جرام موجبة (34) عزلة (37.8٪)، Staphylococcus epidermidis عزلات (6) (6.7٪) و Streptococcus pyogenes عزلات (1) (1.2٪).

- جرثومة صبغة الجرام السلبية تليها Pseudomonas aeruginosa عزلات (6) (7.8٪) ، Escherichia coli و Klebsiella pneumoniaeعزلات(4) (4.5٪) ، Enterobacter cloacaeعزلات(3) (3.4٪) ، بروتيوس ميرابيليس و Acinetobacter ursingiiعزلات (1)(1.2٪).

- العوامل الفطرية 15 (16.7٪) من 90 عينة لها ثلاثة أجناس هي الرشاشيات النيجر (1) (1.2٪)، داء المبيضات الشلل (10) (11.2٪) و Malassezia furfur(4)(4,5٪)

اختبار تأثير العزلات البكتيرية على مجموعة من المضادات الحيوية. وجدت في هذه الدراسة أن المضادات الحيوية سيفترياكسون (73.5٪) والإريثروميسين (70.5٪) أكثر فاعلية ضد العزلات الجرثومية الإيجابية لصبغة جرام، أميكاسين (85.7٪) والجنتاميسين (71.4٪) أكثر فاعلية ضد العزلات الجرثومية السلبية لصبغة جرام.

أظهرت نتائج الفحص المناعي لبعض البارامترات المناعية لمصل المريض فروق معنوية مقارنة مع مجموعة التحكم في هذه الدراسة، كان متوسط تركيز IL-10 لمستوى مصل المرضى ± 0.9 ± SD 28.59 بيكوغرام / مل انخفاضاً معنوياً. مقارنة مع مجموعة التحكم يعني ± 1.9 ± SD 43.07 بيكوغرام / مل خاصة في الأسباب الفطرية يعني ± SD 27.82 (1.8 ±مجم / لتر، في أسباب غير معروفة يعني ± 1.5 ± SD 27.12 مجم / مل والأسباب البكتيرية تعني ± 1.6 ± SD 29.90 ملغم / لتر، مقارنة مع مجموعة التحكم يعني ± SD 43.07 ± 1.9b mg / l.

كان متوسط تركيز IFN-لمستوى مصل المريض (68.35 ± 2.6) بيكوغرام / مل زيادة غير معنوية مقارنة بمجموعة التحكم (61.93 ± 2.5) بيكوغرام / مل خاصة في مجموعة الأسباب

الفطرية 75.03 ± 5.5 بيكوغرام / مل، مجموعة الأسباب غير المعروفة 70.04 ± 5.2 بيكوغرام / مل وزيادة كبيرة في مجموعة الأسباب البكتيرية 64.68 ± 3.4 بيكوغرام / مل مقارنة بمجموعة التحكم 61.93 ± 2.5 جزء من الغرام / مل.

وأظهرت الدراسة أن الجهاز المناعي للمرضى قد تأثر بالعدوى البكتيرية التي سجلت أعلى معدل للمرض.

متوسط تركيز المستضد 4 (CTLA-4)السام للخلايا البشرية السامة للخلايا البشرية في المرضى 42.99 ± 1.4 نانوجرام / مل مقارنة بالتحكم 34.03 ± 1.7 نانوجرام / مل. كانت زيادة معنوية $P < 0.05$ خاصة في مجموعة الأسباب الفطرية 43.79 ± 2.2 نانوجرام / مل ومجموعة الأسباب البكتيرية 42.48 ± 1.8 نانوجرام / مل مقارنة مع مجموعة التحكم 34.03 ± 1.7 نانوجرام / مل.

أظهرت الدراسة أن الجهاز المناعي للمرضى قد تأثر بالعدوى البكتيرية الفطرية في IFN-γ IL-10 وCTLA-4، بينما في IL-10 أظهر تأثر الجهاز المناعي للمرضى بالعدوى البكتيرية في التي سجلت أعلى معدل للمرض